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ISOLATION AND FUNCTIONAL ANALYSIS OF C. ELEGANS INTEGRIN Bpat-3
by

SONYA N. GETTNER

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NEUROBIOLOGY

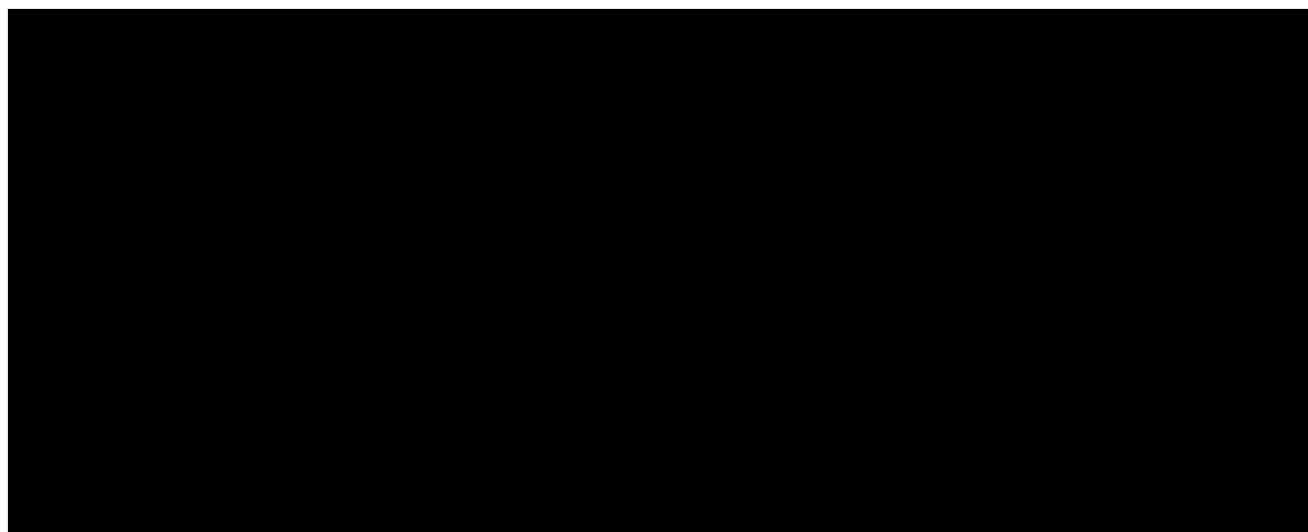
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ACKNOWLEDGMENTS

This thesis, with its tables, figures, and pages and pages of scientific writing, cannot adequately portray the seven years of hard work, suspense, frustration, and the occasional joy of an exciting result that were part of obtaining a doctoral degree at UCSF. Nor can it accurately represent the contributions of the many people who helped make this endeavor a success, sharing with me not only their insights about "doing science", but also their warmth, love, and encouragement. These interactions have been invaluable to me.

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ISOLATION AND FUNCTIONAL CHARACTERIZATION OF *CAENORHABDITIS ELEGANS*
INTEGRIN β pat-3, A NOVEL INTEGRIN β SUBUNIT

Sonya N. Gettner

ABSTRACT

The morphogenesis of tissues requires the adhesion of cells to each other and to the extracellular environment. Integrins are cell surface receptors that mediate cell-cell and cell-extracellular matrix adhesion. The question addressed by this study is: What is the function of integrins during development?

To study integrin function *in vivo*, an integrin β subunit was cloned from the nematode *Caenorhabditis elegans* using the polymerase chain reaction. The amino acid sequence of the *C. elegans* integrin β subunit is conserved in the ligand binding region and in the cytoplasmic domain; in addition, all 56 cysteines characteristic of integrin β subunits are conserved. At least nine proteins co-precipitate with the β subunit; all exhibit characteristics observed in identified integrin α subunits. Immunocytochemical analysis showed that the β subunit is expressed at sites of attachment in muscle, in the somatic gonad, in coelomocytes, and in neurons.

The integrin β subunit gene maps to the left arm of chromosome III. Of the mutants located in this region, *pat-3(rh54)* (paralyzed at two-fold) was a strong candidate for an integrin mutant because it has phenotypes consistent with known integrin function, including defects in cell attachment, cell morphogenesis, process outgrowth, and cell migration. The *C. elegans* integrin β subunit gene, when injected into *pat-3(rh54)*, rescued these defects. In addition to *pat-3(rh54)*, which is an embryonic lethal allele, two viable alleles, *pat-3(rh96)*, and *pat-3(rh151)*, were studied. The mutations in all three alleles were located in the coding sequence. Genetic experiments suggest that *pat-3(rh96)* is an antimorph; that is, the *rh96* mutant protein can interfere with normal β pat-3 function. Immunoprecipitations from *pat-3(rh151)* showed that this mutation affects the association of the putative α subunits. Double mutant combinations of *pat-3(rh151)* with *unc-52*, which encodes a perlecan homolog, revealed a genetic interaction between the two genes, suggesting that Unc-52 is a ligand of β pat-3-containing receptors.

These studies have provided an understanding of integrin β pat-3 at the molecular,

biochemical, and cellular levels and have shown that integrin function is required for many developmental processes.

Jack W. Hall

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CHAPTER ONE

INTRODUCTION

During embryogenesis, the undifferentiated cells generated by early embryonic cell divisions undergo a dramatic reorganization to produce the cohesive tissues and distinct organs of the adult. This reorganization is driven by morphogenetic movements that include the segregation of cells into tissue-specific groups, concerted movements of cell sheets, and long-range cell migrations. In addition, cells differentiate to form tissues and organs and neurons send out projections to synapse with their targets. Cell-cell and cell-extracellular matrix adhesion play a crucial role in these morphogenetic events. One of the current challenges in the field of developmental biology is to determine which molecules are involved in cell adhesion and to elucidate how these molecules are regulated to produce differentiated structures.

In the 1930's, Holtfreter developed a culture system which permitted the survival of dissociated embryonic amphibian cells (Holtfreter, 1938). He observed that dissociated cells initially reaggregated into a spheroid mass by non-selective contact between neighboring cells, but over a period of a few hours, cells sorted out in a tissue specific manner due to active movements of the cells within the spheroid mass (Holtfreter, 1943). Furthermore, he found that individual cells exhibited intrinsic properties of invagination, stretching or spreading, depending on the tissue of origin. These observations led to his proposal of "stage-conditioned positive or negative affinities" between cells (Holtfreter, 1943). In 1946, Tyler proposed that the selective recognition between cells involved the reaction of molecules at the cell surfaces with specificity analogous to the binding of antibodies to antigens (Tyler, 1946). Subsequently, Moscona set up an assay to quantitate the reaggregation of dissociated cells in culture, and found that aggregation of cells was sensitive to temperature (Moscona, 1961). He concluded that the aggregation of cells required metabolic energy, and therefore was an active process rather than a passive process, as had been previously supposed. In 1963, Trinkhaus observed that dissociated cells from early *Fundulus heteroclitus* gastrulae flattened *in vitro* at the same time as epidermal cells in control embryos flattened during the onset of epiboly (Trinkhaus, 1963). He postulated that the flattening of the cells was an intrinsic property of the cell and due to an increase in the adhesive properties of the cell to the substratum. Having also postulated that the movement of the cell sheet was due to the flattening of the cells, Trinkhaus concluded that the movement of the cell sheet was due to an increase in adhesion, thereby linking the cell shape changes directly to changes in adhesivity of the cell (Trinkhaus, 1963).

Over the last decade, two main approaches have been used to identify proteins involved

in cell-cell and cell-extracellular matrix adhesion. The first approach has used immunoselection, in which antibodies that specifically block cell-cell or cell-matrix adhesion subsequently become reagents to identify the receptors. The second approach involves affinity chromatography, in which extracellular matrix proteins are used to purify corresponding receptors. Using these methods, numerous cell surface adhesion receptors have been identified and subsequently classified into several distinct families based on molecular and biochemical criteria (Takeichi, 1987; Hynes, 1987; Reichardt and Tomaselli, 1991; Geiger and Ayalon, 1992; Hynes and Lander, 1992). These families include the cadherins and the integrins, which mediate Ca^{2+} -dependent adhesion, and members of the Ig superfamily such as N-CAM, which mediate Ca^{2+} -independent adhesion (Edelman and Thiery, 1985). As cadherins and integrins were of particular interest in this study, they are described in greater detail in the following section.

The cadherin family of cell surface adhesion receptors.

Members of the cadherin family of cell surface receptors mediate Ca^{2+} -dependent cell-cell adhesion (Takeichi, 1987), binding to receptors on adjacent cells in a homophilic fashion (Nagafuchi et al., 1987) and to a lesser extent, in a heterophilic fashion (Volk et al., 1987). At least eight members of the cadherin family have been well-characterized (reviewed in Geiger and Ayalon, 1992) and additional members have been described (Suzuki et al., 1991). Significant levels of homology exist between cadherins; five homologous repeats have been defined in the extracellular domain, and short regions of highly conserved sequence have been found in the extracellular and cytoplasmic domains. The cytoplasmic domain is not always necessary for Ca^{2+} -dependent aggregation; T cadherin, which lacks a cytoplasmic domain and is anchored to the membrane by a glycosyl phosphatidylinositol glycan, can mediate aggregation when transfected into L cells (Ranscht et al., 1991).

Cadherins are localized in adherens-type junctions in many cell types (Boller et al., 1985; Geiger, 1989; Hirano et al., 1987; Volk and Geiger., 1986 a, b). The co-localization of cadherins with actin filaments, α -actinin, vinculin, catenins, and other components of the cytoskeletal network (Geiger and Ginsberg, 1991) suggests a role for cadherins in providing a transmembrane link to the cytoskeleton. In support of this hypothesis, the cytoplasmic domain of E cadherin, in a complex with α and β catenin, has been shown to bind actin (Ozawa et al., 1990; McCrea and Gumbiner, 1991).

A role for cadherins in morphogenetic processes has been inferred from studies examining the expression patterns of cadherins. For example, changes in cadherin expression are correlated with the formation of the neural tube and the lens: cells in the neural groove and the lens placode, which are the presumptive neural tube and lens, respectively, lose expression of E-cadherin and express N-cadherin instead (Duband et al., 1987; 1988). Shortly after, N-cadherin-expressing cells fold away from the rest of the epithelial sheet, pinching off to form the neural tube and lens. The physical mechanism by which this process occurs has not been completely elucidated, but it may be due in part to the asymmetric contraction of the junctional microfilament belt (Duband et al., 1987; 1988). Additionally, neural crest cells have been observed to lose N-cadherin expression just prior to leaving the neural tube, but re-express it when re-aggregating at their final destination (Duband et al., 1988; Duband, 1990). These observations have suggested that cell-type specific expression of cadherins may lead to the segregation of cells into distinct tissues. *In vitro* studies have substantiated this prediction: when L cells transfected with either E- or P-cadherin are mixed together, the cells sort out in a homotypic fashion (Nose et al., 1988). Furthermore, when L cells transfected with E-cadherin are mixed together with reconstituted embryonic lung tissue, transfected cells associate with lung epithelial cells that endogenously express E-cadherin (Nose et al., 1988).

Studies examining the function of cadherins *in vitro* have shown that cadherins can produce morphological changes within single cells. For example, mesenchymal cells and L cells transfected with E cadherin differentiate and form a polarized epithelial sheet (Ekblom, 1989; Hatta et al., 1988; Nagafuchi and Takeichi, 1988), and N-cadherin has been shown to mediate neurite outgrowth on a variety of substrates, including myotubes (Bixby and Zhang, 1987), astrocytes (Tomaselli et al., 1988), and optic axons (Matsunaga et al., 1988). In addition, results from *in vivo* perturbations experiments support a role for cadherins in morphogenetic events. Injection of anti-E-cadherin antibodies into mouse morulae disrupts blastomer compaction (Hyafil et al., 1980) and injection of antisense oligonucleotides against EP-cadherin into *Xenopus* embryos resulted in the disruption of adhesion between blastomers (Heasman et al., 1994). In addition, function blocking antibodies against N-cadherin disrupt chondrogenesis in the chick limb bud (Oberlender and Tuan, 1994). How cadherins are regulated to produce these morphogenetic events remains to be determined.

The integrin family of cell surface adhesion receptors

Integrins comprise a major class of adhesion receptors that mediate functions such as cell attachment, cell migration, and neurite outgrowth (Hynes, 1992). Originally defined in different systems by monoclonal antibodies generated against cell surface antigens, it was recognized that members of the very late antigen (VLA) family (Hemler, 1988), the leukocyte glycoproteins (LFA-1, Mac-1, and p150,95) (Kishimoto et al., 1987a), the *Drosophila* position specific (PS) antigens (Leptin et al., 1987; Bogaert et al., 1987), and the vertebrate fibronectin receptors (Pytela et al., 1985; Tamkun et al., 1986) had several biochemical properties in common. Most significantly, the complexes contained a β subunit with a molecular weight of approximately 95-120 kDa non-reduced, and one or more non-covalently associated α subunits with molecular weights ranging between 125-200 kDa non-reduced. Each receptor was shown to be composed of a single α subunit and β subunit (Hynes, 1987); thus, β subunits associate with multiple α subunits, but always in pairwise combinations. In addition, a subset of integrin α subunits have been observed to associate with more than one β subunit (Dedhar and Gray, 1990; Hynes, 1992). To date, eight distinct vertebrate integrin β subunits ($\beta 1$ -8) and two *Drosophila* β subunits (β_{PS} and β_v) have been cloned (Hynes, 1992; Yee and Hynes, 1993), and at least 14 distinct vertebrate integrin α subunits and two *Drosophila* integrin α subunits have been cloned or identified (Hynes, 1992; Palmer et al., 1993).

Molecular characterization of cDNAs encoding the integrin subunits has confirmed that both integrin α and integrin β subunits are transmembrane glycoproteins with relatively large extracellular domains and short (40-50 amino acids for the β subunits) cytoplasmic domains. Integrin $\beta 4$ is a notable exception: its cytoplasmic domain is comprised of over 1000 amino acids (Suzuki and Naitoh, 1990; Hogervorst et al., 1990). Co-transfection of integrin α_{IIb} and β_{IIIa} subunits lacking transmembrane and cytoplasmic domains resulted in the expression of functional heterodimers, suggesting that the sequences necessary for dimerization are present in the extracellular domain (Bodary, et al., 1991; Dana et al., 1991; Wippler et al., 1994). Consistent with this observation, molecular characterization of the mutations that cause the human genetic diseases leukocyte adhesion deficiency (LAD) and Glanzmann's thrombasthenia, has revealed that mutations located in the extracellular domain of the integrin $\beta 2$ and $\beta 3$ subunits, respectively, result in the failure of the integrins to dimerize and be expressed on the surface of cells (Kishimoto et al., 1987, 1989; Arnaout et al., 1990; Wardlaw et al., 1990; Loftus et al., 1990; Burk et al., 1991; Bajt et al., 1992).

Each integrin subunit has structural features that define it as an α or β subunit. The majority of integrin β subunits have 56 cysteines in the extracellular domain, most of which are grouped in four cysteine-rich repeats near the transmembrane domain (Fitzgerald et al., 1987). Evidence from electron microscopic analysis (Carrell et al., 1985; Nermut et al., 1988) and biochemical studies has shown that the intramolecular disulfide bonds produce a compact structure at the N-terminal region of the β subunit (Calvete et al., 1991). When electrophoresed under reducing conditions, integrin β subunits characteristically migrate with decreased mobility due to the reduction of disulfide linkages in the extracellular domain (Hynes, 1987). A putative ligand binding region has been identified in the N-terminal half of β subunits by cross-linking studies with the Arg-Gly-Asp (RGD) peptide. This tri-peptide sequence is found in fibronectin, vitronectin, and other integrin ligands of integrin α IIb/ β 3 (Hynes, 1992). In addition, an NPXY motif, which is involved in the internalization of the low density lipoprotein (LDL) receptor (Chen et al., 1990), is present in most, but not all, integrin β subunit cytoplasmic domains. However, this sequence is not required for internalization of the fibronectin receptor, integrin α 5 β 1 (Vignoud et al., 1994).

Integrin α subunits can be divided into subgroups based on various molecular and biochemical criteria. All α subunits have 3-4 cation binding motifs in the extracellular domain, consistent with the observation that integrin-mediated adhesion is divalent cation-dependent. In addition, members of one subgroup of α subunits contain an insert of approximately 180 amino acids, called the I domain, N-terminal to the metal binding motifs (Hynes, 1992). The function of the I-domain is not well understood, but recent studies have implicated a function for this region in ligand binding (Billsland et al., 1994; Kamata et al., 1994). Members of a second subgroup of integrin α subunits are post-translationally cleaved into two disulfide-linked chains. Upon reduction, these α subunits dissociate into a heavy chain containing the majority of the extracellular domain, and a light chain (Hynes, 1987). Integrin α subunits can also be grouped according to which integrin β subunit(s) they associate with; for example, α 1 - α 8 and α v associate with β 1, while α L, α M, and α X associate with β 2 (Hynes, 1992).

In addition to binding extracellular matrix proteins, integrins bind to the cell surface counter-receptors ICAM-1, ICAM-2, and VCAM-1 (Staunton et al., 1988, 1989). The ligand specificity of integrin receptors is determined by the particular combination of α and β subunits (Hynes, 1992). Individual receptors can bind to more than one ligand and, conversely, a ligand can be recognized by more than one integrin receptor, although not

always at the same binding sites within the ligand (Hynes, 1992). Additionally, the specificity of the receptor can depend on the type of cell in which it is expressed: $\alpha 2\beta 1$ expressed in platelets binds specifically to collagen (Staatz et al., 1989), whereas in other cells, this receptor binds to both laminin and collagen (Elices and Hemler, 1989; Kirchhofer et al., 1990a). Additional complexity in ligand specificity may arise from alternate splicing; the *Drosophila* $\alpha PS2$ and βPS subunits are alternatively spliced near the regions of each subunit thought to be involved in ligand binding (Brown et al., 1989; Zusman et al., 1993). The alternatively spliced forms of *Drosophila* βPS have been shown to be functionally significant (Zusman et al., 1993). Finally, integrin receptor specificities can depend on the type of divalent cations present (Kirchofer et al., 1990b, 1991), or the lipids in which they are solubilized (Conforti et al., 1990; Hermanowski-Vosatka et al., 1993).

The affinities of integrin receptors can also be modulated post-translationally. In platelets and leukocytes, respectively, the affinities of $\beta 3$ and $\beta 2$ integrin heterodimers can be activated and deactivated, resulting in specific enhancement of adhesion during events such as platelet aggregation and neutrophil extravasation from the blood stream. Of necessity, this adhesion must be reversible as unactivated cells circulate in the blood stream in order to locate sites where their function is required. The activation of integrins on platelets and leukocytes has been well documented (reviewed in Hemler, 1990; Phillips, 1991; Hynes, 1992; Hynes and Lander, 1992); however, the actual events leading to the activation of integrins have not been fully elucidated. Initially, $\beta 2$ - and $\beta 3$ -containing heterodimers are unable to bind to their ligands, but after activation of the cells by agonists, thrombin or collagen in the case of platelets and inflammatory agents in the case of leukocytes, the receptors become capable of binding their ligands or counterreceptors. This activation involves a conformational change of the receptor demonstrated by the presence of activation-dependent epitopes (Coller, 1985; Shattil et al., 1985; Altieri and Edgington, 1988; Gulino et al., 1990; O'Toole et al., 1991; Andrieux et al., 1991) or by biophysical techniques (Parise et al., 1987; Sims et al., 1991). This conformational change can also be driven by the binding of antibodies (Gulino et al., 1990; O'Toole et al., 1991a; Kouns et al., 1990; Frelinger et al., 1991; Neugebauer and Reichardt, 1991) and is intrinsic to the receptor as it can be produced in purified receptors (O'Toole et al., 1991a). Interestingly, the cytoplasmic domain of integrin α subunits may be involved in regulating the conformational state of the receptor: deletion of the cytoplasmic domain of integrin αIIb , which associates with $\beta 3$ in platelets, results in a constitutively active receptor (O'Toole et al., 1991b). This conformational-dependent

activity may have a role in integrin-mediated developmental functions such as cell migration and axonogenesis.

In addition to mediating transitory adhesion, integrins mediate stable attachment of cells via focal contacts and adherens type junctions. Cytoskeletal proteins such as α -actinin, talin, and vinculin co-localize with integrins at these sites (Chen et al., 1985; Damsky et al., 1985), and talin and α -actinin and have been shown to bind to the cytoplasmic domain of integrins (Horwitz et al., 1986; Otey et al., 1990). Thus, it is thought that integrins provide a link from the extracellular environment to the actin cytoskeleton via these proteins (Tamkun et al., 1986).

Studies of integrin function *in vitro* have implicated a role for integrins in developmental events. These include roles in the differentiation of mesenchyme into polarized epithelium (Sorokin et al., 1990) and the extension of neurites on extracellular matrix proteins (Bozycsko and Horwitz, 1986; reviewed in Reichardt and Tomaselli, 1991) and cell surfaces (Tomaselli et al., 1988). To date, few studies have examined the role of integrins during development *in vivo*. Injection of RGD peptides into chick embryos have implicated integrins in the spreading of the blastoderm on the vitelline membrane (Lash et al., 1990), injection of anti- $\beta 1$ integrin antibodies into embryos have shown that integrins are involved in *Pleurodeles* gastrulation (Darribere, et al. 1988, 1990) and chick neural crest migration (Bronner-Fraser, 1986), and anti-sense RNA experiments have implicated a role for integrin $\beta 1$ in chick neuroblast migration in the tectum (Galileo et al., 1992). In *Drosophila*, the PS integrins have been shown to be required for muscle attachment, dorsal closure, and constriction of the gut in the embryo, and for wing morphogenesis and organization of photoreceptors in the retina post-embryonically (Wright, 1960; Newman and Wright, 1981). Clearly, much work remains to be done to elucidate the functions of integrins during development.

C. elegans as a model system for studying developmental processes

The nematode *Caenorhabditis elegans* has several features which make it particularly well suited for the study of developmental processes. Of primary importance, the anatomy of *C. elegans* is relatively simple and has been well characterized at the light microscope and electron microscope level (Ward et al., 1975; Albertson and Thomson, 1976; White et al., 1976; Sulston and Horwitz, 1977; Sulston et al., 1980; White et al., 1986). The major anatomical structures include the pharynx, the intestine, four longitudinally oriented

quadrants of body wall muscle used for locomotion, dorsal and ventral nerve cords, a nerve ring in the anterior of the animal, a pre-anal ganglion in the tail, and in the adult hermaphrodite, a bi-lobed gonad containing an ovary and a spermatheca on each arm, a uterus, and a vulva (Wood, 1988). These structures are composed of a small number of cells; for example, the somatic gonad, which is the most complex structure in the animal, contains a total of 143 nuclei (Wood, 1988). The axon trajectories and patterns of connection of each of the 302 neurons in the adult hermaphrodite has been completely reconstructed from serial sections analyzed by electron microscopy (Albertson and Thomson, 1976; White et al., 1976; White et al., 1986; Hall and Russell, 1991).

Using differential interference contrast (DIC or Nomarski) microscopy, the nuclei of each cell, the major anatomical structures and the outline of a subset of cells can be visualized in living animals (Sulston, 1976). Because the generation time of *C. elegans* is short, growing from an embryo to a fertile adult in 3 days, developmental events requiring cell adhesion such as cell migrations and morphogenesis of anatomical structures can be observed in real time. The complete pattern of cell divisions, cell deaths, and cell migrations has been determined in the embryo and in post-hatch hermaphrodites and males (Sulston and Horvitz, 1977; Sulston et al., 1980; Sulston et al., 1983). These events have been found to be invariant from animal to animal, resulting in the knowledge of the ancestry of each cell in the developing animal.

C. elegans embryogenesis occurs in approximately 800 minutes, with most of the cell divisions, cell migrations, and organogenesis taking place in the first 400 minutes (Sulston et al., 1983). After 400 minutes, neural processes grow out and synapse with their targets, the embryos elongate, increasing in length in the eggshell approximately four fold by hatching, and a cuticle is secreted. After hatching, the animals go through four larval stages (L1-L4) that are separated by molts of the cuticle. In the third and fourth larval stages, the gonad rapidly increases in size and the sexual structures differentiate (Wood, 1988). Animals enter an alternate third larval stage, called dauer, when subjected to overcrowding or food limitation early in larval development (Wood, 1988). Dauer larvae have a specialized cuticle, do not feed, and have an extended life span.

Due to the short generation time, *C. elegans* is highly amenable to genetic studies (Brenner, 1974). Numerous mutants with phenotypes observed by dissecting microscope or Nomarski microscopy have been isolated in screens of animals treated with common

mutagens such as ethylmethanesulfonate (EMS) (Wood, 1988). Thus, the *in vivo* consequences of loss-of-function and other genetic mutations can be studied with single cell resolution in living animals. The small size of the genome (10^8 bp) has facilitated the cloning of greater than 95% of the genome into an ordered array of cosmids and yeast artificial chromosomes (YACs) (Coulson et al., 1986). The cloning of genes for identified mutations has resulted in linking the genetic and physical maps. As a result, for most genetic locations, overlapping cosmids and YACs are available from the corresponding region of the physical map. Likewise, *C. elegans* genes cloned by homology with genes isolated from other organisms can be positioned on the existing physical map and the approximate location on the genetic map determined. As a consequence, the genetic map may be examined to determine whether any previously isolated mutations, which may be located in the gene of interest, exist in the region.

What is the role of cell surface adhesion receptors in C. elegans development?

The major objective of this study was to determine the role of cell surface adhesion receptor function in *C. elegans* development using *C. elegans* genetics. The experiments performed to fulfill this objective are presented in the following two chapters. In Chapter 2, the question of whether homologs of known vertebrate adhesion receptors could be cloned from *C. elegans* was addressed (see also appendix I), and experiments examining the molecular and biochemical properties of an integrin β subunit that was cloned are presented. In Chapter 3, the role of this integrin in *C. elegans* development was determined by the identification and analysis of a mutation in the integrin β subunit gene. The conclusions of this study are presented in the final chapter.

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CHAPTER TWO

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF β pat-3, A NOVEL INTEGRIN β SUBUNIT ISOLATED FROM *C. ELEGANS*

SUMMARY

Members of the integrin family of cell surface receptors have been shown to mediate a diverse range of cellular functions requiring cell-cell or cell-extracellular matrix adhesion, including cell attachment, migration, differentiation, and neurite outgrowth. Because these processes occur during the development of all animals, we were interested in cloning integrin receptors from the nematode *Caenorhabditis elegans* in order to study integrin function in individual cells *in vivo*. Here we report the cloning of an integrin β subunit from *C. elegans*. In the following chapter, the integrin β subunit gene is shown to rescue the mutation *pat-3(rh54)*; thus, the integrin β subunit cloned in this chapter has been named *C. elegans* integrin β pat-3. Analysis of the deduced amino acid sequence revealed that *C. elegans* integrin β pat-3 is most homologous to the *Drosophila* integrin β PS and to the vertebrate integrin β 1. Regions of highest homology are in the ligand binding region and in the cytoplasmic domain; in addition, the 56 cysteines present in the majority of integrin β subunits are conserved. A major transcript of approximately 3 kilobase pairs was detected by RNA blot analysis. Immunoblot analysis with a polyclonal antiserum generated against a peptide corresponding to the cytoplasmic domain showed that *C. elegans* integrin β pat-3 migrates with an apparent Mr of 109 kDa in SDS-PAGE under non-reducing conditions and 120 kDa under reducing conditions. At least nine protein bands with relative molecular weights in the range observed for known integrin α subunits co-precipitate with *C. elegans* integrin β pat-3, and at least three of these bands migrate in SDS-PAGE with increased mobility when reduced. This behavior has been observed for a majority of integrin α subunits. Immunoprecipitations of *C. elegans* integrin β pat-3 from developmentally staged populations of *C. elegans* showed that the expression of several of the co-precipitated bands changes during development, suggesting developmental regulation of integrin $\alpha\beta$ heterodimers. The monoclonal antibody MH25, which has been postulated by Francis and Waterston (Francis and Waterston, 1985) to recognize the transmembrane component of dense bodies in *C. elegans* body wall muscles, was shown to recognize *C. elegans* integrin β pat-3. Finally, immunocytochemical analysis revealed that *C. elegans* integrin β pat-3 is expressed in the embryo and in many cell types post-embryonically, suggesting an important role for the integrin in the developing animal.

INTRODUCTION

The interactions of cells with their environments are critical for the development and maintenance of tissues in multicellular organisms: the processes of proliferation, migration, differentiation, and regeneration are dependent on soluble factors, proteins in extracellular matrix, and proteins expressed on the surfaces of cells. Cell surface receptors play an important role in these processes by detecting information in the extracellular environment and transmitting this information into the cell. Members of one family of cell surface receptors, the integrins, have been shown to mediate biological processes requiring cell-cell or cell-extracellular matrix adhesion such as cell attachment, cell migration, and neurite outgrowth. Ligands of integrin receptors include the extracellular matrix components laminin, collagen and fibronectin, and the cell surface proteins ICAM-1, ICAM-2 and VCAM-1. The ligand specificity of an integrin receptor is dependent upon its subunit composition: each receptor is a heterodimer composed of non-covalently associated α and β subunits (Hynes et al., 1989). To date, fourteen α subunits and nine β subunits have been identified (Hynes, 1992). Distinct complements of subunits are expressed in different tissues and cell lines, determining in part which heterodimers form. Additional complexity arises from the observation that the ligand specificity of a particular heterodimer can depend upon the cell type in which it is expressed (Staat et al., 1989; Ellices and Hemler, 1989; Kirchhofer et al., 1990a). Furthermore, studies of integrin-mediated platelet aggregation have revealed that the adhesive functions of integrins can be regulated by second messengers (Wright and Silverstein, 1982), by ligand binding (Du et al., 1991) and by binding of antibodies (Keizer et al., 1988; Neugebauer and Reichardt, 1991; van Kooyk et al., 1991). Experiments using monoclonal antibodies and biophysical techniques (Sims et al., 1991), have shown that the conformation of an integrin receptor changes concurrently with receptor activation. In relatively static structures such as focal adhesion plaques, integrins are thought to provide a transmembrane link between the extracellular matrix and the actin cytoskeleton via vinculin, talin and α -actinin (Chen et al., 1985; Damsky et al., 1985). However, for integrin-mediated cell migration and neurite outgrowth, it is not yet clear whether these proteins interact with integrins and how this interaction is regulated to produce motility.

In vitro studies and immunocytochemical analyses have implicated a role for integrins in developmental processes; however, few experiments have addressed the role of integrins during development *in vivo*. In chicken, injection of an antibody that blocks the function

of $\beta 1$ -containing heterodimers into embryos revealed that integrins are required for neural crest cell migration (Bronner-Fraser, 1986). In *Pleurodeles* embryos, injection of anti- $\beta 1$ antibodies was found to inhibit gastrulation (Darribere et al., 1988, 1990). To date, the analysis of the *Drosophila melanogaster* integrin β PS subunit mutant *lethal(1)myospheroid* has provided the most complete information about integrin function during development (Wright, 1960; Newman and Wright, 1981; Leptin et al., 1989; Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990). *Lethal(1)myospheroid* is an embryonic lethal mutation that has defects in muscle attachment, dorsal closure, and constriction of the gut. Post-embryonic phenotypes of *lethal(1)myospheroid* include defects in wing morphogenesis and organization of photoreceptors in the retina. Surprisingly, no phenotypes due to defects in cell migration or axon outgrowth were observed, although subtle phenotypes may have been difficult to detect.

To understand the role of integrins during development with greater resolution than provided by previous studies, we have chosen to study the function of integrins in the nematode *Caenorhabditis elegans*. *C. elegans* is a particularly attractive experimental animal for the study of integrin function for several reasons. First, the cellular and subcellular localization of integrins can be determined with exquisite detail because the identity and lineage of each somatic cell is known from the embryo to the adult (Sulston and Horvitz, 1977; Sulston et al. 1980; Sulston et al. 1983). Second, because *C. elegans* is amenable to genetic manipulations (Brenner, 1974), mutations in integrins can be isolated and the resultant phenotypes studied with single cell resolution in the living animal by differential interference contrast (DIC or Nomarski) microscopy. Third, *C. elegans* genetics can be used to identify and isolate genes whose products interact with the integrins. Finally, it is of interest to learn whether integrin function is conserved through evolution. In this chapter, we describe the cloning of a *C. elegans* integrin β subunit, the primary sequence of its cDNA and the organization of its gene. This gene was shown to rescue the *C. elegans pat-3(rh54)* mutation in the following chapter; thus, it will be referred to as *C. elegans* integrin β pat-3. A polyclonal antiserum prepared against a peptide corresponding to the cytoplasmic domain of *C. elegans* integrin β pat-3 was used for immunoblot analysis, immunoprecipitations and immunocytochemistry. The immunoprecipitations showed that at least nine protein bands co-precipitate with *C. elegans* integrin β pat-3; these bands have relative molecular weights in the same range as known integrin α subunits. Immunocytochemical analysis showed that *C. elegans* integrin β pat-3 is first expressed in the embryo and is subsequently widely expressed in larvae and adult animals. The complex pattern of the *C. elegans* integrin β pat-3 expression and

MATERIALS AND METHODS

Polymerase chain reaction (PCR) amplification of an integrin β subunit from C. elegans

Degenerate oligonucleotides for use in the polymerase chain reaction (PCR) were designed from the deduced amino acid sequences of human integrin β 1, β 2, and β 3, chicken integrin β 1, *Xenopus laevis* integrin β 1, and *Drosophila melanogaster* integrin β PS. The oligonucleotide primers were designed according to the following criteria:

- 1) Regions of high homology (at least 6 consecutive amino acids identical or conserved) between the amino acid sequences of the integrin β subunits were selected for designing the primers.
- 3) The conserved regions were no greater than 500 nucleotides apart to minimize the likelihood that an intron would be contained in the intervening genomic sequence.
- 4) The degeneracy of the oligonucleotides was no greater than 1024-fold.
- 5) Codon usage for *C. elegans* was used to decrease the degeneracy of the oligonucleotide sequences; no more than two potential mismatches were allowed per oligonucleotide.
- 5) Oligonucleotide primers were between 17 and 22 nucleotides in length.

The temperatures for the annealing and extension cycles of the PCR were lowered from the recommended temperature for Taq polymerase to maximize annealing of the oligonucleotide primers to template DNA. Thus, the conditions for PCR were: denaturing temperature, 94°C for 1 min; annealing temperature, 45°C for 1 min; extension temperature, 55°C for 3 min; forty cycles were run in a Perkin Elmer thermocycler. The reaction mixes for PCR contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1 μ g of each primer, 1 μ g of *C. elegans* strain N2 genomic DNA, and 0.62 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Oligonucleotide primers for PCR were synthesized at the oligonucleotide synthesis facility in the Howard Hughes Medical Institute at UCSF.

Oligonucleotide primers designed from the integrin β subunit ligand binding region,

β #7 (5'GA(CT)CT(N)TA(CT)TA(CT)(CT)T(N)ATGGA3')

β #8 (5'TC(N)AC(AG)AA(N)GA(N)CC(AG)AA(N)CC3')

where N=A,C,G and T, were successful in amplifying a DNA fragment of the predicted size.

The amplified DNA was subcloned and sequenced to verify that the protein predicted from the DNA sequence internal to the primers was homologous to integrin β subunits.

Isolation of C. elegans integrin β pat-3 genomic and cDNA clones

2×10^4 plaques from a *C. elegans* strain N2 λ EMBL3 genomic library were screened. Nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH) lifted from the plates were prehybridized and hybridized in a modified Church buffer (0.5 M sodium phosphate, pH 7.2; 7% SDS; 1 mM EDTA; 1% BSA; 10% formamide) at 55°C (Church and Gilbert, 1984) and washed with 2x SSC with 0.1% SDS at 50°C. The library was screened with radiolabeled DNA prepared from a gel-purified insert containing the PCR-amplified β pat-3 sequence. The DNA was uniformly labeled with ^{32}P -dCTP using Klenow (Boehringer/Mannheim, Indianapolis, IN) and oligonucleotides β 135#7 and β 135#8 to synthesize the second strands. Seven hybridizing clones were purified.

5×10^5 plaques from a *C. elegans* strain N2 2-3 kb size-selected ISHLX2 cDNA library provided by Dr. Chris Martin were screened as described above, except the prehybridization and hybridization temperature was 50°C. The radiolabeled DNA used to screen the cDNA library was prepared from a gel-purified 5 kb Sal-1 fragment derived from phage clone (2-4) isolated in the genomic library screen. The DNA fragment was radiolabeled with ^{32}P -dCTP using oligonucleotide β 135 3' as a primer for the synthesis of the second strand. Eleven hybridizing clones were isolated.

Subcloning and sequencing

Restriction enzyme mapping by single and double restriction enzyme digests and Southern blot analysis of the seven genomic phage clones using the radiolabeled probe described for the genomic library screen were carried out according to Maniatis et al., 1982. A 5 kb Sal-1 fragment was subcloned from genomic phage clone (2-4) and partially sequenced using oligonucleotide primers β 135 5' and β 135 3' to verify that the clone contained sequence homologous to integrin β subunits. A 9 kb Sal-1 fragment from genomic phage clone (1-1) which hybridized with oligonucleotides from the 5' (β 135-PE) and 3' (β 135-7rev) ends of the cDNA sequence was subcloned and sequenced to determine intron and non-coding 5' and 3' sequence.

The lengths of the cDNA inserts were determined by PCR amplification of the insert DNA using the T7 and SP6 oligonucleotide primers and sequences complementary to the T7 and SP6 oligonucleotide primers flanking the insert. cDNA phage clone 1-1 contained the longest insert and was therefore sequenced completely. The 5' ends of the other clones

were sequenced and found to contain sequence identical to that found in cDNA 1-1.

PCR-amplified DNA, cDNA inserts and DNA fragments from the genomic phage clones were subcloned into M13, pIBI (IBI, New Haven, Ct), or Bluescript (Stratagene, La Jolla, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) or Boehringer/Mannheim. The nucleotide sequence of cDNA clone 1-1 was determined from a single-stranded template derived from M13; both DNA strands were sequenced. The nucleotide sequences of the PCR products and the genomic clones were determined from double-stranded templates. The PCR-amplified DNA, cDNA 1-1, and the exon/intron borders in the 9 kb Sal-1 fragment from genomic phage clone 1-1 were sequenced using the dideoxy chain termination method of Sanger et al., 1977, and reagents from the Sequenase kit (U. S. Biochemicals, Cleveland, OH). Intron sequence was determined by the Biomolecular Resource Center DNA sequencing facility (UCSF) using a Perkin Elmer Cetus PCR System 9600 for DNA amplification and an Applied Biosystems 370A DNA sequencer. Gene-specific oligonucleotide primers for sequencing were synthesized at the Biomolecular Resource Center and the oligonucleotide synthesis facility in the Howard Hughes Medical Institute at UCSF.

DNA and Protein Sequence Analysis

DNA and amino acid sequence analysis was performed using PCGene (IntelliGenetics). Alignments of the amino acid sequences of integrin β subunits was performed using the Pattern-Induced Multi-sequence Alignment (PIMA) algorithm (Smith and Smith, 1987; 1991). Sequences in between cysteines not aligned using the PIMA algorithm were aligned using the alignment program in PCGENE.

RNA blot analysis

Total cellular RNA was prepared from mixed populations of worms using the method of Rosenquist and Kimble (1988). Poly A⁺ RNA was prepared from total RNA by batch processing with oligo dT (Collaborative Research, Bedford, MA). RNA was electrophoresed in formaldehyde-containing agarose gels according to Maniatis et al., 1982, transferred onto a Hybond-N membrane (Amersham, Arlington Heights, IL) using 20x SSC, and crosslinked to the membrane with ultraviolet light. The membrane was prehybridized and hybridized in a modified Church buffer (0.5 M sodium phosphate, pH 7.2; 7% SDS; 1 mM EDTA; 1% BSA; 30% formamide) and washed with 1x SSC with 0.05% SDS at 55°C. cDNA 1-

1 was radiolabeled with ^{32}P -dCTP using random primers (Boehringer/Mannheim) to probe the RNA blot. The relative molecular weight of the *C. elegans* integrin $\beta\text{pat-3}$ transcripts was determined by comparison with RNA molecular weight standards purchased from Gibco/BRL (Gaithersburg, MD).

Generation of peptide antiserum

The deduced amino acid sequence of the cytoplasmic domain of *C. elegans* integrin $\beta\text{pat-3}$ was analyzed by the PCGENE antigenicity program (IntelliGenetics, Mountain View, CA) to predict the most antigenic portion of the cytoplasmic domain. A peptide corresponding to this sequence (CKWDTNENPIYKQATTTFKNPVYAGKAN; the 5' C was added for coupling) was synthesized in the Howard Hughes Medical Institute at UCSF. The peptide was coupled to keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., San Diego, CA) as follows: 9 mg of sulfo-MBS (Pierce, Rockford, IL) was added to 18 mg of KLH in 50 mM potassium phosphate, pH 7.0 and rocked for 1 hour at 4°C. Activated KLH was purified away from unreacted sulfo-MBS by fractionation on a G-25 (Pharmacia LKB Biotechnology, Inc., Piscataway NJ) column and combined with peptide (1:7) in 50 mM potassium phosphate, pH 7.0 overnight at 4°C. The KLH-coupled peptide was sent to Caltag Laboratories (South San Francisco, CA) for immunizations into sterilely bred, pathogen-free rabbits. To affinity purify the antiserum, IgGs precipitated with ammonium sulfate and resuspended and dialyzed in 10 mM potassium phosphate, pH 7.8 before purification on a DEAE-cellulose (DE-52, Whatman, Clifton, NJ) column. Fractions containing IgG were pooled and run over a column containing the *C. elegans* integrin $\beta\text{pat-3}$ cytoplasmic peptide coupled to thiopropyl-Sepharose 6B (Pharmacia). Thiopropyl-Sepharose 6B was prepared according to the manufacturer's directions and coupled to the peptide by rocking overnight at 4°C in 0.1 M sodium acetate, pH 4.8. Before adding the IgG fractions, the peptide-coupled thio-Sepharose 6B was washed with PBS followed by 0.1 M glycine, pH 2.7 to remove any uncoupled peptide; the washes were repeated twice in this order. After the final glycine wash, the peptide-coupled thio-Sepharose was washed once in PBS and once in (10 mM potassium phosphate, pH 7.8; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5). The IgG fraction was added to the peptide-coupled thio-Sepharose 6B, supplemented with NaCl and Tris-HCl, pH 7.5 to final concentrations of 150 mM and 50 mM, respectively, and coupled overnight with agitation at 4°C. Non-specific antibodies were washed from the column with 10 mM potassium phosphate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5. Specific antibodies were eluted with 0.1 M glycine pH 2.7, immediately neutralized with 1 M Tris-HCl, pH 8.3 and concentrated using a Centricon 100 microconcentrator (Amicon, Danvers, MA). The

affinity-purified antibody, which will be referred to as the β pat-3 cytoplasmic antibody, was used for all of the experiments.

Animals

C. elegans strain Bristol (N2) worms were used for all experiments with the exception of the dauer sample in the developmentally-staged immunoprecipitation. To ensure a pure population of dauers, the temperature-sensitive dauer-constitutive mutant *daf-2(e1370)* was used. N2 and *daf-2(e1370)* worms were propagated at 20°C on agar in petri dishes seeded with *Escherichia coli* strain OP50 as a food source according to Brenner (1974).

To stage worms, 1-3 mls of a mixed population of worms containing a high percentage of gravid hermaphrodites were dissolved with bleaching solution (4-6% sodium hypochlorite:4 M NaOH:H₂O; 3:1:1) to release embryos (Emmons et al., 1979). The embryos were hatched onto plates containing OP50 and the plates examined using a dissecting microscope to determine the approximate stage of development of the worms. For each sample collected, 100 worms were checked by Nomarski microscopy to determine the percentage of worms of each stage. To obtain dauers, *daf-2(e1370)* embryos were placed in the presence of food at the restrictive temperature of 25°C and the hatchlings were monitored until dauers appeared on the plates. Subsequently, the *daf-2(e1370)* worms were kept at 25°C for an additional 24 hours to allow the entire population to enter the dauer state.

Worms were collected for the experiments in M9 buffer (Wood, 1988) and pelleted in a clinical centrifuge setting at medium speed; pellets were frozen in a dry ice/ethanol bath and stored at -80°C if not used immediately.

Extraction of proteins from worms

To extract protein, 1 ml of ice cold Western lysis buffer (20 mM Tris-HCl, pH 7.5; 0.32 M sucrose) or immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM MgCl₂; 1 mM CaCl₂; 1% Triton X-100 for radiolabelled proteins or 1x PBS/0.1 M HEPES, pH 7.0 with 0.32 M sucrose for proteins to be labeled with biotin) supplemented with freshly added proteinase inhibitors (50 µg/ml final chymostatin, leupeptin, aprotinin, and pepstatin; 1 mM PMSF (Sigma, St. Louis, MO) or AEBSF (ICN Biochemicals, Cleveland, OH) and 0.5 ml white quartz sand (Sigma) was added per 0.2-0.5 ml pellet of

worms. The worms were vortexed at maximum speed for 45 sec alternating with 30 seconds on ice until most of the worms were lysed (approximately 20 minutes). The extract was transferred to an eppendorf tube and spun at low speed (1000 G) at 4°C for 2 minutes to remove debris. The supernatant was transferred to a fresh eppendorf tube and spun at 2500 G at 4°C for five minutes to remove nuclei and cytoskeleton.

Immunoblot analysis

For immunoblot analysis of *C. elegans* integrin β pat-3, protein extracts were spun at high speed (15,000 G) at 4°C for 30 min to pellet membranes. The membranes were resuspended in 6x Laemmli buffer and the concentration of protein determined by the Amido Schwartz method. The samples were electrophoresed through a 6% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Schleicher and Schuell). The nitrocellulose was blocked with Blotto (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5% Carnation dry milk); incubations with antibodies were also done in Blotto for 1 hr at RT. The β pat-3 cytoplasmic antibody, MH25, and the *C. elegans* integrin β pat-3 cytoplasmic peptide were diluted to a final concentration 10 μ g/ml. Alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Promega, Madison, WI) were diluted 1:7500. The blots were washed 3x for 5 min with Blotto after each antibody incubation and 1x with alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.0; 0.1 M NaCl; 5 mM $MgCl_2$) prior to incubation with NBT (50 mg/ml) diluted 1:150 and BCIP (50 mg/ml) (Sigma) diluted 1:300 in alkaline phosphatase buffer.

Radiolabeling of worms

To label worm proteins with ^{35}S , worms were fed radiolabeled bacteria prepared as follows: OP50 bacteria were grown to saturation in LB at 37°C and diluted 1:100 in low-sulfate M9 (M9 containing 0.5 mM magnesium sulfate) supplemented with 0.1% glucose, 1 mM uracil, and 0.01 mg/ml thiamine. The culture was grown again to saturation, diluted 1:100 in low sulfate medium a second time and grown until a density of $OD_{600}=0.2$ was reached. At this point, 500 μ Ci of ^{35}S -methionine, 500 μ Ci of ^{35}S -cysteine, and 200 μ Ci of $^{35}SO_4$ (New England Nuclear, Boston, MA) were added to the culture; the culture was returned to 37°C until saturation was reached. The radiolabeled bacteria were pelleted, resuspended in 2 ml of low-sulfate M9 and plated onto 2 10 cm NG agar plates. The plates were left at RT overnight to allow the bacteria to grow to confluency. 200 μ l of a mixed population of worms were placed on each plate until no trace of bacteria remained. The

worms were shaken gently in M9 for 15 min and washed 3x in M9 before protein was extracted.

Non-radioactive labeling of worm protein extract

Worm protein extracts were labeled with sulfo-NHS-biotin (Pierce) as an alternative to labeling with ^{35}S . Before labeling, the protein extract was spun at high speed (15,000 G) to pellet membranes. The supernatant was discarded and the pellet resuspended in 1 ml of 1x PBS/0.1 M HEPES, pH 7.0 supplemented with proteinase inhibitors. 20 μl of 10 $\mu\text{g}/\text{ml}$ freshly prepared sulfo-NHS-biotin were added to the protein extract; the extract was incubated at 4°C for 2 hours with rocking. To stop the labeling reaction and remove unreacted biotin, 100 μl of 1 M Tris, pH 8 was added to the extract and the membranes were pelleted at 15 krpm at 4°C for 25 min and washed 3x with 1x PBS/0.1 M Tris, pH 8, supplemented with PMSF or AEBSF. After the final wash, the membranes were solubilized in 1 ml ice cold immunoprecipitation buffer (100 mM Tris-HCl, pH8; 150 mM NaCl; 1 mM MgCl_2 ; 1 mM CaCl_2 ; 1% Triton X-100) plus PMSF or AEBSF. The extracts were precleared with 100 μl of protein A sepharose (Pharmacia) for 1 hour at 4°C with rocking followed by a preclear step with 100 μl of protein A sepharose plus 30 μl of preimmune serum or non-immune serum for 1 hour at 4°C.

Immunoprecipitations

To immunoprecipitate $\beta\text{pat-3}$, a protein extract from radiolabeled worms or the precleared supernatant from a biotinylated protein extract were divided into the same number of eppendorf tubes as samples (200-500 $\mu\text{l}/\text{sample}$) and 20 μl of serum or 20 μg of purified antibodies were added to each sample. The samples were rocked at 4°C overnight. The samples were precleared 2x for 1 min with 75 μl of Sepharose Cl-4B beads (Pharmacia) before incubation with 75 μl of protein A Sepharose Cl-4B beads (Pharmacia) at 4°C for 45 min. After the incubation, the beads were washed 5x with an excess volume of wash buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM MgCl_2 ; 1 mM CaCl_2 ; 0.2% SDS, 0.1% Triton X-100 for radiolabeled samples; 100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM CaCl_2 , 1% Triton X-100, 0.2% SDS for biotinylated samples).

Reciprocal immunodepletions of biotinylated protein extracts using MH25 and the $\beta\text{pat-3}$ cytoplasmic antibody were performed as described above except that the extract was incubated 4-5x with the depleting antibody and protein A sepharose before

immunoprecipitation with the second antibody.

To visualize the immunoprecipitated proteins, the protein extract- β pat-3 cytoplasmic peptide antibody-protein A sepharose complexes were resuspended in 6x Laemmli buffer, boiled for 3 min, and the supernatant electrophoresed through a 6% SDS-polyacrylamide gel. Gels containing radiolabeled samples were stained with Coomassie blue, destained, dried and sprayed with En³Hance (New England Nuclear) before exposure to Kodak X Omat AR film (Rochester, NY). Biotinylated proteins were electroblotted onto nitrocellulose and the blots blocked for 1 hr at RT with 10% bovine serum albumin, fraction V (Sigma) and 0.05% Tween-20 before incubation for 1 hr at RT with alkaline-phosphatase conjugated streptavidin (Accurate, San Diego, CA) diluted 1/50,000 or with HRP-streptavidin (Zymed, South San Francisco, CA) diluted 1/4,000 in 1% bovine serum albumin, fraction V plus 0.05% Tween-20. Blots incubated with alkaline-phosphatase conjugated streptavidin were washed 3x 5 min at RT with 1x PBS pH 7.0 and developed as described above. Blots incubated with HRP-streptavidin were washed 1x 5 min at RT with (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), 2x 5 min at RT with (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton-X-100), and 2x 5 min at RT with (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and developed with ECL reagents (Amersham) according to the manufacturer's instructions. Relative molecular weights of the proteins were determined by comparison with molecular weight standards purchased from Bio-Rad (Richmond, CA).

Two-dimensional SDS-PAGE of immunoprecipitated biotinylated proteins was carried out as follows. In the first dimension, samples were electrophoresed through a 6% SDS-polyacrylamide gel under non-reducing conditions. Non-reduced prestained markers (Sigma) were included with the immunoprecipitate to visualize the location of the sample in the gel. After electrophoresis, a strip of gel containing the sample was incubated in 2x Laemmli buffer with 10% β -mercaptoethanol for 1 hr at RT followed by 5 min on top of a tempblock set at 100°C. For electrophoresis in the second dimension, the gel strip was rotated 90°C and placed at the top of the gel plates, the gel plates sealed, and a 4% SDS-polyacrylamide stacking gel followed by a 7.5% SDS-polyacrylamide gel poured on top of the strip. The sample was electrophoresed through the gel, electroblotted and developed for the alkaline phosphatase reaction as described above. Biotinylated molecular weight standards (Biorad) pre-incubated with iodoacetamide and iodoacetamide added to a final concentration of 2.5% were also included in the sample.

Immunocytochemistry

Worms were prepared for immunocytochemistry using the freeze/crack method according to Wood et al. 1988. Briefly, 10-12 μ l of worms in distilled water were placed on microscope slides freshly coated with 0.1% poly-L-lysine and allowed to settle. After a coverslip was placed on top of the worms, the slide was placed under a dissecting microscope for viewing and liquid withdrawn with bibulous paper until the worms were immobile. The slides were then frozen on dry ice for a minimum of 5 min, the coverslip popped off with a razor blade, and the slides stored at -80°C until used for staining. The fixation of the animals consisted of an incubation for 5 min in either -20°C methanol or -20°C ethanol, followed by 1 min in -20°C acetone and three washes in 1x PBS, pH 7.0 for 10 min. The slides were blocked for 1 hr at 37°C with 5% bovine serum albumin, fraction V, 5% normal goat serum (NGS) in 1x PBS, pH 7.0, before incubation for 2 hr at 37°C with the β pat-3 cytoplasmic antibody diluted 1/200 or MH27 diluted 1/500 in 5% BSA/5% NGS in 1x PBS, pH 7.0. The slides were washed 3x in an excess volume of 1x PBS, pH 7.0 before incubation for 1 hr at 37°C with TRITC-conjugated goat anti-rabbit or anti-mouse antibodies (Accurate), diluted 1/500 in 5% BSA/5% NGS in 1x PBS, pH 7.0. The slides were washed 3x for 10 min in 1x PBS, pH 7.0 and mounted for viewing with 80% glycerol containing 2% n-propyl gallate, and 1 μ g/ml diamidinophenolindole (DAPI) using a Zeiss Axioscope microscope equipped with fluorescence optics. All images were photographed onto Kodak Techpan film and printed on Agfa paper (Leverkusen, Germany) at high contrast.

RESULTS

Cloning of an integrin β subunit from C. elegans

Using degenerate oligonucleotide primers designed from the deduced amino acid sequence of the ligand binding region of human integrin $\beta 1$, human integrin $\beta 2$, human integrin $\beta 3$, chicken integrin $\beta 1$, *Xenopus* integrin $\beta 1$, and *Drosophila* integrin βPS , a DNA fragment of the expected size was amplified from *C. elegans* genomic DNA by PCR. The amino acid sequence predicted from the *C. elegans* DNA amplified between the primers contained an open reading frame with sixteen of the thirty two amino acids (50%) identical to the human $\beta 1$ sequence (Fig. 1). To clone more of the gene, the PCR-amplified DNA was used to screen a *C. elegans* genomic library. A 5 kb Sal-1 fragment derived from one of the genomic phage clones isolated in the screen was partially sequenced to confirm that it contained an integrin β subunit gene. The sequence analysis revealed an open reading frame containing 114 codons with thirty-one of seventy four predicted residues (42%) identical with the human integrin $\beta 1$ sequence. A 9 kb Sal-1 fragment containing the entire coding region of the integrin β subunit was shown to rescue the *C. elegans pat-3(rh54)* mutation in the following chapter; therefore, the integrin β subunit cloned in this study has been named *C. elegans* integrin $\beta pat-3$.

Coding sequence analysis

To obtain the full-length coding sequence of *C. elegans* integrin $\beta pat-3$, a cDNA library was screened using a probe prepared from the 5 kb Sal-1 fragment. Sequence analysis revealed that the longest cDNA isolated, cDNA 1-1, encodes a transcript of 2908 nucleotides in length. The 5' end of the cDNA contains 11 nt of the 22 nt *C. elegans* SL1 sequence, which is a leader sequence *trans*-spliced from an unlinked exon onto approximately 10% of *C. elegans* transcripts (Krause and Hirsch, 1987). After the SL1 sequence, 20 nt precede the proposed initiator methionine and a large open reading frame of 2,427 nt. The 3' untranslated sequence is 451 nt in length and contains a stretch of 41 adenosines at the 3' end. On the basis of computer analysis and comparisons with other integrin β subunits, the predicted protein is composed of 809 residues and has a signal peptide of 19 amino acids, an extracellular domain of 719 amino acids, and a transmembrane domain of 49 amino acids. The predicted molecular weight of the mature protein is 88,120 daltons; with an additional 22,500 Daltons from nine potential N-linked glycosylations (average 2,500 Daltons/N-linked glycosylation), the predicted molecular

Figure 2-1. Alignment of the deduced amino acid sequence of the PCR fragment amplified from *C. elegans* genomic DNA with the corresponding sequences from previously reported integrin β subunits. Highly conserved regions from the deduced amino acid sequence of the ligand binding regions of *Drosophila* β PS, *Xenopus* β 1, chicken β 1, human β 1, human β 2, and human β 3 (shown in boxes) were used to design degenerate oligonucleotides for PCR. The sequences of the oligonucleotides are shown above the regions from which they were designed; arrows indicate positions where *C. elegans* codon usage was used to decrease the degeneracy of the oligonucleotide. The deduced amino acid sequence of the PCR fragment amplified from *C. elegans* genomic DNA is shown aligned with the corresponding sequences from the other integrin β subunits; amino acids conserved between the *C. elegans* sequence and more than 4 of the other sequences are shaded. The amino acid sequence predicted from the oligonucleotide sequence in the PCR fragment is indicated by dashes as it does not represent the true *C. elegans* sequence.

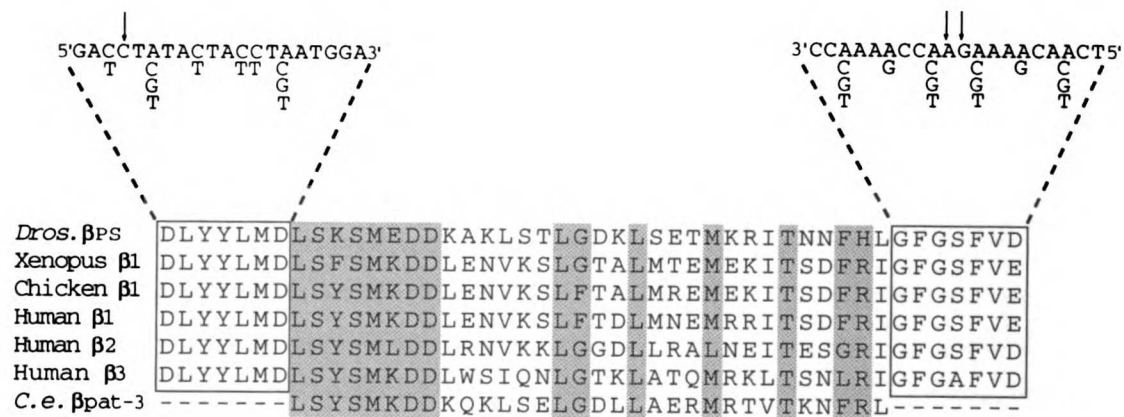


Figure 2-2. Nucleotide sequence and deduced amino acid sequence of the *C. elegans* integrin β pat-3 subunit. Coding DNA sequence from cDNA 1-1 is shown in upper case letters; non-coding sequence is shown in lower case letters. The partial SL1 *trans*-splice leader sequence (nucleotides 1-11) is highlighted by a fine underline. The regions corresponding to the oligonucleotides used for PCR are marked by a dashed underline. Downward triangles indicate the positions of the seven introns. The amino acid sequence is shown in the single-letter code below the second nucleotide of each codon. The putative signal peptide cleavage site (between residues 19 and 20) is marked by an arrow and the predicted transmembrane sequence (residues 738-760) is highlighted by a heavy underline. Cysteines are indicated by dots under the amino acid sequence, and the nine potential *N*-linked glycosylation sites are denoted by asterisks.

gcaagtttgagttgaacctaccggcatcaaATGCCACCTTCAACATCATGTGCTCCTCGCAGCACTTCTTCCATTGCTTTACCAGCAAGCGATTGGAAGAC 105
M P P S T S L L L L A A L L P F A L P A S D W K T 25

TGGAGAAGTCACTGGAAAAGTCGTCGAAAAATCAGAATTCCTCATGTACAGTCTGTACAGTGACAATTACACATGCTCAGCGTGCATTCAATATCATGAATCTTG 210
G E V T G K V V E K S E F P C Y S L S R D N Y T C S A C I Q Y H E S C 60

CGCGTGGTGGAGCACCGATGTTTCGACGAGAAGAAGCCATATGCCCGTTGTATAGCCGTGCCAAGCTCATGGAACATGGATGTCCAAACAGTTATATCGAGGA 315
A W C G A P M F D E K K P Y A R C D S R A K L M E H G C P N S Y I E D 95

TCCGGCCACAAAATTTGGATATCACTGAAGACAGTAACTCTCCGATCAAGGACAAGTTGAATCTGAGGAAGAAGCCGTTTCAGATCAAACACAGGAGATGTACGT 420
P A T K L D I T E D S K L S D Q G Q V E S E E E A V Q I K P Q E M Y V 130

CGAAATTCGGCCAAATCCCGTGTTCGTTTCAATGTGACCTACCGTCAAGCCGTTGATTATCCAGTTGACTTGTACTATTTGATGGAATCTCTCATATTCATGAA 525
E I R P K S R V R F N V T Y R Q A V D Y P V D L Y Y L M D L S Y S M K 165

GGATGCAAAACAGAACTGTCCGAACCTCGGAGACTTGTCTCGCGAAGCAATGAGAAGTGTCAAAAGAATTTCCGATTAGGATTCGGTTTCATTGACAAGAA 630
D D K Q K L S E L G D L L A E R M R T V T K N F R L G F G S F I D K K 200

ACTTATGCCATTTCATCGATCCACGAATCGAAAAACAATTATCTCCGTGTCCAACACCATGTGCAAGACCATATGGATTCAAGCATCAATGTCAATGACGACGAA 735
L M P F I D P R I E K Q L S P C P T P C A E P Y G F K H Q M S L T T N 235

CACGGCAAGTTTCAAGGCTGAAGTTGATAAAGCTGAAATTTCTGGAAATTTGGATGTCTCGGAAGGAGGTTTCGATGCTGTTGTTTCAGGCGTTGGCTTGTAAATA 840
T A K F L D I V D K A E I S G N L D I M V D N A N A S E K C N K 270

AACCATTTGGGTGGCGTGAACGTGCTCGTAAATGATCGTTTCTCAACTGACGCTGGATTCCATTTCGCTGGAGACGGAAGATTGGCAGGTGTCTCGAACCAGAA 945
T I G W R E R A R K M I V F S T D A G F H F A G D G R L A G V V E P N 305

TGATGGAACATGTCATTAGATAGAGAAGGATATTACTGAAACACTTAATCAAGATTATCCATCAATTGCACTTCTTCATCAATGATCAAGGATCGTAAGGC 1050
D D T C K L S E L G Y Y T E T L N R T V T K N F R L G F G S F I D K K 340

AAATGTCATTTTTGTGTTTACAAGAACAATCAGGATTGTATACACAATTTGTCGAATGCATCCAGATGTTTCATCGTCGGTTGGAGTTTGGCTAATGATTC 1155
N V I F A V T K N N Q D L Y T Q L S N A L P D V S S S V G V L A N D S 375

AAGAAATATTGTCGATTGATTGAGAAGGAATTTGAAAAATAGTGAAGATCATTATGGTTGATAATGCAATGCTTCAGAAGGACTCAAGCTCACTTACAG 1260
R N I E V D L K I S E K I M V D N A N A S E K C N K 410

ATCTATGTGTTTGGAGAACCACTGAAAGATACCAATGTTTGTGAGGAATTCGTGTTGGAGATGAAGTTCAATTCGAAGTAACCTTGAAAACTACTCATTG 1365
S M C L D G T T L K D T N V C E G I R V G D E V Q F E V T L E N T H C 445

TATCGATAAAGTGTATTTGTTCTCCGTATCGGACCATCTGGATTGGATGAGACATTCATGTTAATGTTAAAGTTTATGCGATTGTCGATGTAACGTCAAGA 1470
I D K R I G P S G L D E T L I V N V K V L C D C D C E R Q D 480

CAGAATTGTGACGAATTCGGCTGATTGTAACGAGGAGATATGGTTTGTGAGTTTGTGATGCAAGGAGGAAATGTTGGAATATTTGTAATGTAATAGACC 1575
R I V T N S A D C N G G D M V C G V C R C K G G N V G K Y C E C N R P 515

TGGAATGAGTACTGCTCGCTCAATGAAAAATGCAAAAGAACTAACGAATCAGCAATCTGTGAGGTCGTGGTGTGTGTAATCTGGACGTTGTGAATGTAATCC 1680
G M S T A A L N E K C K R T N E S A I C E G R G V C N C G R C E C N P 550

ACGTGCCAATCCAGAAGAACAAATCTCTGGAGAATTCGTGAATGCGCAACTTCAATTGCCACGACACGATCGTAAATCTGTGCGAAGACCGGTGAATGCAA 1785
R A N P E E Q I S G E F C E C D N F N C P R H D R K I C A E H G E C N 585

CTGTGGAAGTGTATTTGTGCACCTGGATGGACTGGAAGAGCCTGTGAATGCCAAATTTCAACTGATTCAATGCCTCTCTGCAAAATGGAATAATCTGTAATGGA 1890
C G K C I C A P G W T G R A C E C P I S T D S C L S A N G K I C N G K 620

GGGTGAATGTATTTGTGGAAGATGTCGATGCTTCGATTGCGCCGACGGAATTCGATATTCGGAGCGAAATGCGAAATTTGTCCGACGTGTCGACGAAATGTGT 1995
G E C I C G R C R C F D S P D G N R Y S G A K C E I C P T C P T K C V 655

GGAATACAAGAATTGTGTAATGTGCCAGCAATGGCAGACAGGCCACTTAATGAGACCGCTGTGATCAGTGTGAATTCAAAGTTATTCTGTGTGAGGAATTACC 2100
E Y K N C V M C Q Q W Q T G P L N E T A C D Q C E F K V I P V E E L P 690

CAATCTCAACGAACTACACCTGCCAATTTGTGGATCCAGCTGATGATTGTACATCTTATCTACTATTACGATGAGGCCACAGATAATGCAACAGTCTG 2205
N L N E T T P C Q F V D P A D D C T F Y Y L Y Y D E A T D N A T V W 725

GGTCAGAAAACATAAAGATTGTCTCCACCTGTCCCTGTGCTCGCAATTTGTGCTCGGAGTCATTGCGGGTATCGTAATCTCGGAATTTCTCTGTTGCTCTG 2310
V R K H K D C P P P V P V L A I V L G V I A G I V I L G I L L L L L W 760

GAAATTGCTCAGTACTTTCATGATAGATCCGAATACGCTACTTTCAATAACGAAAGGCTTATGGCCAAATGGGATACGAACGAGAACCCCAATACAAACAGGC 2415
K L L T V L H D R S E Y A T F N N E R L M A K W D T N E N P I Y K Q A 795

CACGACAACATTTAAAAATCCAGTATACGCTGGAAGGCAACTaaatagtttttatccttatattttaataattttcccaaattttctaataatgaagctcaat 2520
T T T F K N P V Y A G K A N 809

tctccatccaaacaactcgaaacgagtagtttagcgataaattgtcacattctctttgttttattcaaaaaatctccattttttgttttttttctcttcatca 2625
attgttcatcacactgcttttaccctattcctaataacggatttttttctctcttatatttgacttgcctttatattcattctttcattgctttttgtccctc 2730
ttaacaccgaccaacaactcacacacttctgtactatttccaattttcaatggtgcttttatttttgaactatcttgttcgaattttttcatgtaatttaattt 2835
tatatgaaaaaatctgaaaaatttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaagggggccc 2908

weight is 110,620. The complete cDNA and deduced amino acid sequences are shown in Figure 2.

Comparison of C. elegans integrin β pat-3 with previously cloned integrin β subunits

Comparison of the *C. elegans* integrin β pat-3 amino acid sequence with human integrins β 1- β 8 and *Drosophila* integrin β PS revealed that *C. elegans* integrin β pat-3 is most homologous to *Drosophila* integrin β PS (44.7% identity), although *C. elegans* integrin β pat-3 does not contain the 40 amino acid insert 5' to the ligand binding region unique to *Drosophila* (Fig. 3). Of the integrin β subunits cloned from human, *C. elegans* integrin β pat-3 is most homologous to β 1 (41.2% identity). The 56 cysteines characteristic of the extracellular domains of integrin β 1, β 2, β 3, β 5 and β 6 are present in *C. elegans* integrin β pat-3 and their spacing is conserved. The ligand binding region, which was originally identified by crosslinking the RGD peptide to the integrin β 3 subunit (D'Souza et al., 1988) and is highly conserved in all integrin β subunits sequenced to date, is highly conserved in *C. elegans* integrin β pat-3. This region extends from D¹⁴⁹ to residue D²⁰⁶ in *C. elegans* integrin β pat-3, and has 60.3% identity with the human β 1 sequence and 69% identity with the *Drosophila* β PS sequence. The cytoplasmic domain of *C. elegans* integrin β pat-3 is also highly conserved, with 59.6% identity with human integrin β 1, 52.5% with human integrin β 3, and 72.3% identity with *Drosophila* integrin β PS, and identities ranging between 23%-45% for human integrins β 2, β 5, β 6, and β 7. A consensus sequence derived from the alignment of human integrins β 1- β 8, chicken, mouse, and *Xenopus* integrin β 1, and *Drosophila* integrin β PS shows that 96 residues in the extracellular domain are invariant (Fig. 3); 41 of these residues are cysteines.

Genomic organization

We were interested in cloning a DNA fragment containing the entire *C. elegans* integrin β pat-3 gene in order to identify an integrin mutant with the injection/rescue method (see following chapter). A 9 kb Sal-1 genomic fragment, which was shown to hybridize by Southern blot analysis with oligonucleotide primers designed from the 5' and 3' ends, respectively, of the cDNA sequence, was subcloned and sequenced to determine the organization of the gene. The genomic sequence is shown in Figure 4. The length of the gene from the first nucleotide of exon 1 to the position at which the genomic sequence diverges from the cDNA sequence is 5,224 nt. The coding sequence is divided into eight exons ranging from 69 nt to 488 nt in length; the seven introns range between

Figure 2-3. Alignment of the *C. elegans* integrin β pat-3 deduced amino acid sequence with the sequences of human integrin β 1, *Drosophila* integrin β PS, and a consensus sequence of integrin β sequences. The deduced amino acid sequences are shown in the one letter code and are numbered starting from the initial methionine. Dashes denote gaps that were introduced into the sequences to maximize the alignment. Residues that are identical between the sequences are shaded. The consensus sequence was derived from the sequences of *C. elegans* β pat-3, human β 1- β 8 (Argraves et al., 1987; Kishimoto et al., 1987a; Fitzgerald et al., 1987; Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Ramaswamy and Hemler, 1990; McLean et al., 1990; Sheppard et al., 1990; Erle et al., 1991; Moyle et al., 1991) chicken β 1 (Tamkun et al., 1986), *Xenopus* β 1 (DeSimone and Hynes, 1988), and *Drosophila* β PS (MacKrell et al., 1988).

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Human β1	MN-----LQPIFWIGLISS-----VCCVFAQTDENRC--LKANAKSCGECIQAGPNC	45
C.e. βpat-3	MPPSTSLLLLAALLPFALPASDWKKTGEVTKGVVEKSEFFPCYSLSRDNYTCSACIQYHESC	60
Dros. βps	MILERNRRCLALLMIAMLAIAAQTNAQKAAKLTAVSTCASKEK----CHTCIQ-TEGC	55
Consensus β	M.....C.....C.....C.....	
Human β1	GWCTNSTPLQEGMPTSSARCDLLEALKKKKGCPDDIENPRGSKDIKKKNKVNTRSKGT---	102
C.e. βpat-3	AWCGAPMF--DEKKPYARCDSSRAKLMEHGCPNSYIEDPATKLDITEDSKLSDQGGQVE---	115
Dros. βps	AWCMQPDF-----KGQSRCYQNTS---SLGPEEFAYSPTVEQILVNKLTNQYKALAA	107
Consensus β	..C.....RC.....	
Human β1	-----AEKLPEDIHQIQPQQLVLRKNS	125
C.e. βpat-3	-----SE----EEAVQIKPKQEMYVEIRP	134
Dros. βps	GGGGSAMSGSSSSSSSYSSSSSSSSSFYSQSSSGSSSASGYEYSAGEIVQIQPSMRLLALRV	167
Consensus β	Q...PQ.....	
Human β1	GEPTFTLKFKAEDYPIDLYYLMDLSSYNKDDLENVKSLGTDLMNEMRRITSDFRIGFG	185
C.e. βpat-3	KSRVRFNVTYRQAVDYPVDLYYLMDLSSYNKDDKQKLSLGDLLAERMRTVTKNFRLGFG	194
Dros. βps	NEKHNIKISYSAQEGYFVLDLYYLMDLSSKSMEDDKAKLSTLGDKLSETMKRITNNFHLGFG	227
Consensus β	P.D.Y.LMD.S.SM.....G..L.....GFG	
Human β1	SPVEKTVMPYISTTPAKLRNPCTSEQNCTTFPSYKNVLSLTNKGVEFVNLVKGQRISGNC	245
C.e. βpat-3	SFIDKKLMPFIDPRIEKQLSPCPTP--CAEPYGFKHQMSLTNTAKPKAEVDKAEISGNC	252
Dros. βps	SFVDKVLMPYVSTIPKLEHPC--E-NCKAPYGYQNHMPLNNNTESFSNEVKNATVSGNC	284
Consensus β	P.....E.....S.M.	
Human β1	DSPEGGFDAIMQVAVCGSLIGWRN-VTRLLVFSTDAAGFHAFAGDGKLGIVLFPNDGQCHLE	304
C.e. βpat-3	DAPEGGFDAVVOALACNKTIGWRERARKMIVFSTDAAGFHAFAGDGRLAGVVEPNDGTCHLD	312
Dros. βps	DAPEGGFDAIMQAIACRSQIGWRREQARRLLVFSTDAAGFHAFAGDGKLGIVLFPNDGECHELS	344
Consensus β	D.PEGG.DA..Q...C...IGWR.....H...D..L.....D..CH..	
Human β1	NNM-YTMTHYDYPSIAHLVQKLSENNIQTIFAVTEEFQPVYKELKNLIPK--SAVGTDS	361
C.e. βpat-3	REGYYTETLNQDYPISIALHQMIDKRRKANVIFAVTKNNQDLYTQLSNALPDVSSSVGVLA	372
Dros. βps	PKGEYTHSTLQDYPISISQINQKVKDANAILIFAVT-ASQ-LSVYEKLVHEHIQSSAAKLD	402
Consensus β	Y.....P.....FAV.....	
Human β1	ANSSNVLIQIIDAYNLSSEVI-LENGKLSEGVTTISYKSYCKNGVNGTGENGRKCSNYSI	420
C.e. βpat-3	NDSENVLDLIEKEYLKIIEKIIMVDNANASEGLKLTYSMC--LDGTTLDKDTNVCCEGIRV	430
Dros. βps	NDSSNVVELVKEEYRKISSSVENKDD--NATGDVKITYFSSC--LSNGPEVQTSKCDNLKE	458
Consensus β	N...L.....	
Human β1	GDEVQFEISITSNKCCKKDSDS-FKIR--PLGFTBEVEVILQYICCEEC--Q-SEGIPES	474
C.e. βpat-3	GDEVQFEVTLNTHC-IDKRD--FVLRIQSGGLDETLLVNVKVLCDCECDCEQDRI-VTNS	486
Dros. βps	GQQVSETAQIQLLKCPEDPRDWTQTIIHISFVGINEVMQIQLTMLCSCPCENPGSIGYQVQ	518
Consensus β	C.....C.C.C.....	
Human β1	P-KCHEGNGTFECGACRCNEGRVGRHCECS-TDEVNSEDMDAYCRKENS-EICSNNGEC	531
C.e. βpat-3	A-DC-NG-GDMVCGVCRCKGGNVGKYCEC-NRPGMSTAALNEKCKRTNES-AICEGRGV	541
Dros. βps	ANSK-SGHGTSMSGICNCDDSYFGNKCECSATDLTSKFANDTSCRADSTSTTDCSGRGH	577
Consensus β	G...G.C.....G..C.C.....C.....C.....C.....C.....	
Human β1	VCGQCVCRRKRDNTNEIYSGKFCEDNFNCNDRSNGLIC-G-GNGVCKERVCECNPNYTGSN	589
C.e. βpat-3	NCGRCECNFRANPEEQISGEFCEDNFNCNDRHDKKIC--AEHGECCNCGKICAPGWTGRN	599
Dros. βps	VCGACECHKRPNIPIEIIISGKHCECDNFSCERNRNQLCSGPDHGTCECGRCKCKPGWTGSN	637
Consensus β	.C..C.C.....G..C..D...C.....C.....C.....C.....G..	
Human β1	CDCLSDTSTC-EASNGQICNGRGICECGVCRK-T--DP-KFQGGTCEMGTCLGLVCAEHE	644
C.e. βpat-3	CECPISTDNC-LSANGKICNGKGECICGRCECFDSDPDGNRYSGAKCEICPTCKVEYK	658
Dros. βps	CGCQESNDTCMPGGGGEICSGHGTCECGVCRK-TVNDQGRFSGRHCEKCEPTCSGRQRL	696
Consensus β	C.C.....C.....C...G.C.C...C.C.....C.....C.....	
Human β1	ECVQCRAFKNKG-KKDT-CTQECSEYFNITKVESRDKLPQVPQDPVS---HCKEKD-VDD	698
C.e. βpat-3	NCVMCQQWQTGPL-NETACDQ-CEFKVIPVEELPN-----LNETT---PCQFVDPADD	706
Dros. βps	DCVQCQMYKTGELKNGDDCARNCTQFVPVGVVEKVE-----IDETKDEQMCKFFD-EDD	748
Consensus β	.G..C.....C.....C.....	
Human β1	CWFYFTYSVN-GNNEVMVHVVENPECPGPDIIPIVAGVAGIVLIGLALLLWKLLEMI	757
C.e. βpat-3	CTFYLLVYDEATDNATVVRKHKDCPPVPVLAIVLGVAGIVILGILLLLWKLLETVL	766
Dros. βps	CKFMFKYS---EQGELHVVYAQENKECPAKVFMGLGIVMGVIAAIVLVGLAILLLWKLLETT	805
Consensus β		
Human β1	HDRREFAKFEKEKMNKWDGTGENPIYKSAVTTVVNPKYEGK	798
C.e. βpat-3	HDRSEYATFNNERLMKWDTHENPIYKQATTTFKNPVYAGKAN	809
Dros. βps	HDRREFARFEKERMNKWDGTGENPIYKQATSTFKNPYAGK	846
Consensus β		

Figure 2-4. Sequence of the *C. elegans* integrin β pat-3 gene. A 9 kB sal-1 fragment from genomic phage clone 1-1 was sequenced to determine the organization of the gene. The sequence shown begins just prior to the first exon and continues past the site in the poly A sequence where the cDNA sequence diverges from the genomic sequence. Exons are highlighted in bold type. Coding sequence is shown in upper case letters; non-coding sequence is shown in lower case letters. Nucleotide numbering is shown in the right margin. Oligonucleotide primers used to sequence the sense strands of cDNA 1-1 and the 9 kB sal-1 fragment are denoted by arrows over the sequence; primers used to sequence the anti-sense strands are shown below the sequence.

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taatnttttcagttgaacctaccgggcatc¹aaATGCCACCTTCAACATCATTTCTCTCTCTCCAGCAGCTTCTTCCATTCTGCTTTACCAAGCAAGCGATTGG 100
 AAGACTGGAGAACTCACTGGAAAGTCGTGAAAAATCAGAATTCCTCATCTGTACAGTGTACAGTGTACAAATTACACATCTCAGCGTGCATTCAATATC
 ATGAATCTTCCGCTGTGTGTGAGCACC^{1,2}CGtatgttaaagcttgagaccatgaatttatttttaataaaaaaattccagATGTTTCGACGAGAAAG^{1,5}
 AGCCATATGCCCTTGTGTATAGCCCTGCCAAGCTCATGGAACTGTGATGTTTCAAAACAGTTATATCGAGGATCCGCCACAAAATTGGATATCATCTGAAAG^{1rev}
 CAOTAACTCTCCGATCAAGGACAAAGTTGAATCTGAAGAAAGAGCCCTTCAGATCAAAACCAAGAGATGTACGTGAAATTCGCCCAgtaagttttt 500
 atatgggttttcaagataattcggttttaaaaactcgatgaaaataatcaataaaatacaaaaaatttttgggggtttcagcaaaaaaacgaagatttcag
 ggtaatt^{1,2}tcgtagtcttaaat¹ttcaggaaaaatttgcactaattaagaaatgtttttcattgatttttcacaacaaaaaccgctgaaaatcacatttg
 aaaaattgtctcaaaaactgaaaaaaaatcgactgcat¹tttttgcggaaagttaaataatttcgggttttggttattttcagcctcttttttatt
 taatntttatcttaaaaagctcctgtaatatacaattaagtgaagtaatttattattattatcataattcggtgatttagacatttttaggctcggtttgac^{1,5}
 tatttttggattttaagagatttttgggcttaaaatgacctaaatcaacgaattaatgataaacttctaaaatttgcaattcagggtgattataaaata^{1,5} 1000
 aaaaataattagtaccgctgcgactcgaaaactgtaaaatcacaagattttctctaaatccagttcttcaatatcaacaacaatctaaatgttttt
 tttcagAAATCCCTGTCTGTTTCAATGTGACCTACCGTCAAGCCGTGATTTATCCAGTTGACTTGTACTATTGATGGATCTCTCATATTCCATGAAAGGA^{1,3,5}
 TGACAAACAGAAATCTCGAACTCGGAGACTTCTCTCGGAAACGAATGAGAACTGTGACAAAGAAATTCGATTTAGGATTCGCTTCAATTCATTGACAAAG^{1,3,5}
 AAATCTTATGCCATTTCATCGATCCAGAAATCGAAAAACAATTATCTCCGTGTCCAAACCATGTGTCAGAACCATATGGATTCAAGCATCAAAATGTCATTGA^{1,3,5}
 CGAGAAACACGGCAAGTTCAAGGCTGAAGTTGATAAAGCTGAATTTCTGGAATTTGGATGCTCCGGAAGGAAGTTTCGATGCTGTTTGTTCAGCCCTT 1500
 GCGTTGTAATgtaagtgggtattttgattttaaagaaaaattcaaaaagaatcaaatcaacatttttgagatcgcgggaagcttaaaatctaaaaatcaa
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 CGGTTGGAATTTTGGCTAAATGATTCAGAAATATTGTGCAATTGATTGGAAGGAATATTGAAAAATTAAGTGAAGAAAGATCAATTATGTTGATTAATGCAAA^{3,5} 2000
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53 nt and 617 nt. The location of the introns with respect to the cDNA and amino acid sequences is shown in Figs. 2 and 5A. The sequence contained in the introns was translated and examined for open reading frames, as alternatively spliced exons have been found in vertebrate integrin β subunits. Although several open reading frames were detected, none were flanked by *C. elegans* consensus splice donor or splice acceptor sequences.

To learn whether the organization of integrin β subunits genes is evolutionarily conserved, the positions of introns in the *C. elegans* integrin β pat-3, human integrin β 2, β 3, β 7 and *Drosophila* integrin β PS genes were compared. The human integrin β 1 gene was not included, as information regarding its genomic organization is incomplete. The comparison revealed that the location of four introns is conserved between the *C. elegans* integrin β pat-3 gene and the human integrin β 2, β 3 and β 7 genes, and the location of two of these four introns is conserved in the *Drosophila* integrin β PS gene. The first 3 introns conserved between the *C. elegans* integrin β pat-3 gene and the human integrin β subunit genes are located in the extracellular domain near the ligand binding region, and the fourth intron conserved in all of the genes examined divides the coding sequence of the cytoplasmic domain in two (Fig. 5B). In all of the integrin β subunit genes examined, the four cysteine-rich repeats of *C. elegans* integrin β pat-3 are not encoded by discrete exons; instead, introns interrupt the second cysteine-rich repeat in the *C. elegans* integrin β pat-3 gene, the first and third cysteine-rich repeats of the human β 2 and β 7 genes, and the third cysteine-rich repeat of the human β 3 gene.

RNA blot analysis

An RNA blot of *C. elegans* total cellular RNA was probed with radiolabeled cDNA 1-1 to confirm that cDNA 1-1 accurately represents the *C. elegans* integrin β pat-3 transcript. A major band of approximately 3 kb in length was observed (Fig. 6A). A band of this size was also detected on an RNA blot containing *C. elegans* total poly A⁺ RNA (Fig. 6B, lane 3). The signal from the poly A⁺ RNA is greatly enriched relative to signals from lanes containing total RNA and RNA not retained on oligo dT (Fig. 6B, lanes 1 and 2, respectively), as expected for a polyadenylated transcript. The signal at the position of the 17s ribosomal RNA appears to be due to non-specific hybridization to ribosomal RNA as it is decreased in the poly A⁺ RNA lane compared with the other lanes. Thus, the RNA blot analysis shows that the lengths observed for the *C. elegans* integrin β pat-3 transcript and cDNA 1-1 are similar.

Figure 2-5. Organization the *C. elegans* integrin β pat-3 subunit gene and alignment of β pat-3 exons with the exons of human β 2, human β 3, human β 7 and *Drosophila* β PS. a) The organization of exons and introns in the integrin β pat-3 gene is shown relative to the predicted structure of the β pat-3 protein. Exons are represented by numbered black boxes and introns by a solid black line. The predicted ligand binding region, cysteine-rich repeats, and transmembrane domain are indicated in the diagram of the β pat-3 protein by shading. The length of the exons and introns in the gene and the position of the exons in the protein are drawn to scale. b) The exons of *C. elegans* β pat-3 are aligned with the exons of human β 2 (Weitzman et al., 1991), human β 3 (Lanza et al., 1990; Zimrin et al., 1990), human β 7 (Jiang et al., 1992) and *Drosophila* β PS (Albert MacKrell, pers. comm.). The positions of the introns are indicated by vertical lines and numbered in order along the top of the protein. Introns conserved between β pat-3 and the other β subunits are highlighted by an asterisk. The size of the β subunits was made uniform to demonstrate the alignment. Introns 1, 2, 3, and 7 of β pat-3 correspond to introns 3, 4, 6, and 15 of human β 2; introns 2, 3, 5 and 14 of human β 3; introns 3, 4, 6, and 15 of β 7. Introns 2 and 7 of β pat-3 correspond to introns 1 and 4 of β PS.

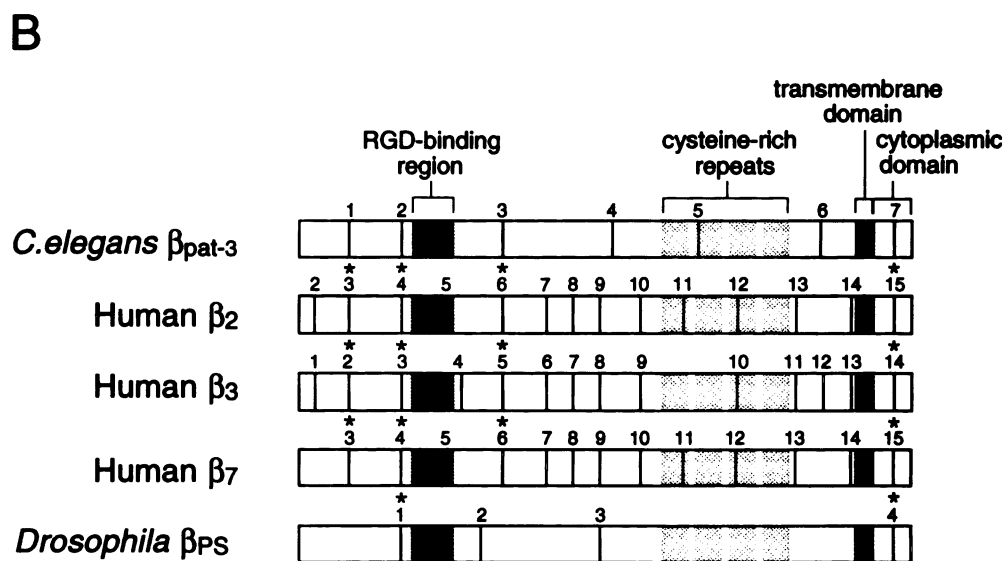
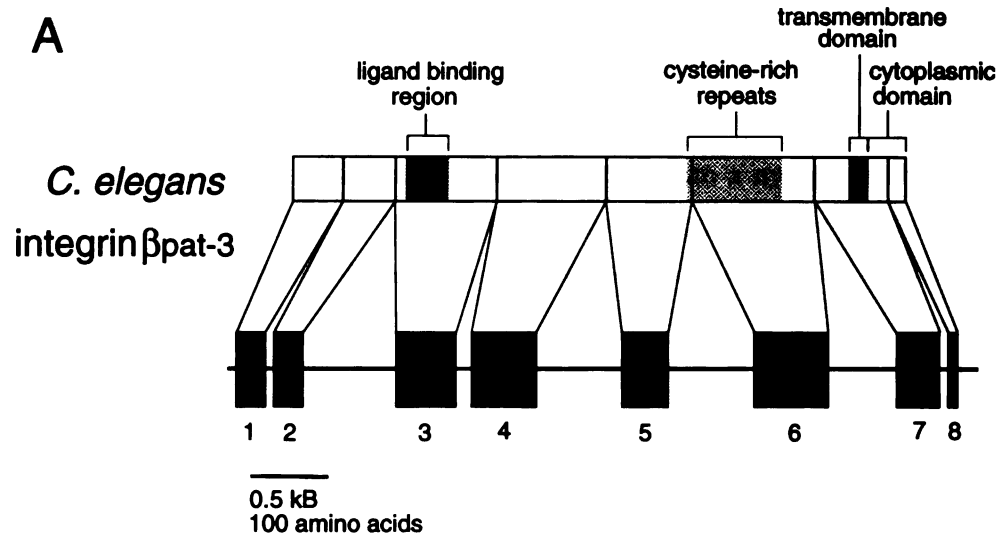
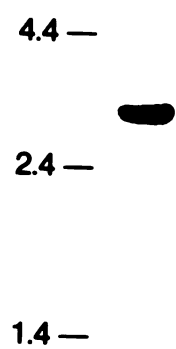


Figure 2-6. RNA blot analysis of *C. elegans* integrin β pat-3 transcripts. a) 50 μ g of *C. elegans* total RNA was separated by electrophoresis, blotted onto a nylon membrane and probed with radiolabeled cDNA 1-1. b) 20 μ g of *C. elegans* total RNA (lane 1), 20 μ g of flow-through RNA from a oligo dT column (lane 2), and 2 μ g of poly A⁺ RNA (lane 3) were separated by electrophoresis, blotted onto a nylon membrane and probed with radiolabeled cDNA 1-1. A major transcript of approximately 3.0 kB was detected. The positions of the molecular weight markers (shown numerically in kilobase pairs) are marked on the left.

A



B

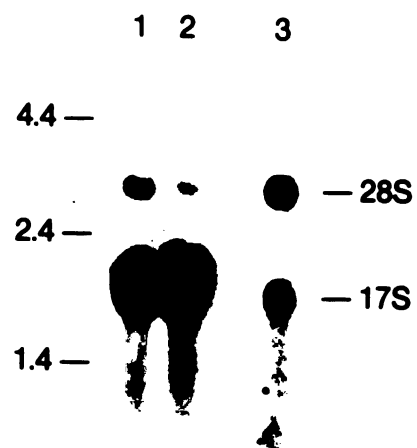


Figure 2-7. Immunoblot analysis of the *C. elegans* integrin β pat-3 protein.

A polyclonal antibody was generated against a peptide corresponding to 27 amino acids of the predicted cytoplasmic domain of β pat-3; the affinity-purified antibody will be referred to as the β pat-3 cytoplasmic antibody. A protein extract was prepared from *C. elegans* N2 worms, separated on a 6% SDS-polyacrylamide gel under non-reducing conditions (lanes 1-3) and under reducing conditions (lanes 4-6), and electroblotted into a nitrocellulose membrane. The nitrocellulose was incubated with preimmune serum (lane 1 and 4), the β pat-3 cytoplasmic antibody (lanes 2 and 5), and the β pat-3 cytoplasmic antibody plus the cytoplasmic peptide (lanes 3 and 6). The β pat-3 cytoplasmic antibody recognizes a band of Mr 109 kDa under non-reducing conditions (lane 2) and a band of Mr 120 kDa band under reducing conditions (lane 5). The numbers at the left indicate the positions and sizes of the molecular weight markers in Daltons $\times 10^3$.

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Characterization of the C. elegans integrin β pat-3 protein

In order to study the *C. elegans* integrin β pat-3 protein, a peptide corresponding to the twenty seven carboxyl-terminal residues of the cytoplasmic domain was synthesized, coupled to a carrier protein, and used to generate a polyclonal antiserum. In immunoblot analysis, the affinity purified antibody (hereafter referred to as the β pat-3 cytoplasmic antibody) recognizes a single band with an approximate Mr of 109 kDa non-reduced (Fig. 7, lane 2) and 120 kDa reduced (Fig. 7, lane 5). This decrease in electrophoretic mobility under reducing conditions as compared with non-reducing conditions has been observed for other integrin β subunits, and is presumably due to the high cysteine content in the protein. No *C. elegans* proteins were recognized by the preimmune serum (Fig. 7, lanes 1 and 4). The specificity of the β pat-3 cytoplasmic antibody was demonstrated by competition with the peptide used to generate the antibody (Fig. 7, lanes 3 and 6).

C. elegans integrin β pat-3 is widely expressed post-embryonically

As a first step towards understanding the function of *C. elegans* integrin β pat-3 during development, the cellular localization of *C. elegans* integrin β pat-3 in whole-mount preparations of worms was determined using the β pat-3 cytoplasmic antibody. Prominent staining was detected throughout development in muscle cells. In the four quadrants of body wall muscle used for locomotion, each of which is composed of two rows of obliquely oriented uninucleate cells, *C. elegans* integrin β pat-3 is localized to the dense bodies, the M-lines, and the borders between cells (Figs. 8A and B). The dense bodies, which are Z-disc analogs, and the M-lines are thought to anchor the thin and thick filaments, respectively, of the sarcomere (Wood, 1988). In contrast to body wall muscles, which have distributed attachment sites, the diagonal muscles of males, which are located in the tail and used to produce the ventral arching movements characteristic of mating behavior, have focal attachments at the ventral and lateral attachment sites of the muscle. *C. elegans* integrin β pat-3 was detected at the lateral attachment site of the diagonal muscles; the ventral site was not examined (Fig. 8C). *C. elegans* integrin β pat-3 was also detected in the anal depressor muscle, which is an H-shaped cell that opens the anus during defecation. *C. elegans* integrin β pat-3 is localized at the ventral attachment site of the muscle, apposing the anus; in addition, staining was detected along the middle strut which straddles the anus (Figs. 8D, E and F). More anteriorly along the intestinal-rectal tract, *C. elegans* integrin β pat-3 was detected on the ventral surface of the sphincter muscle (Figs. 8D and F) and the basal surface of the intestinal muscles (Fig. 8D). In the vulval

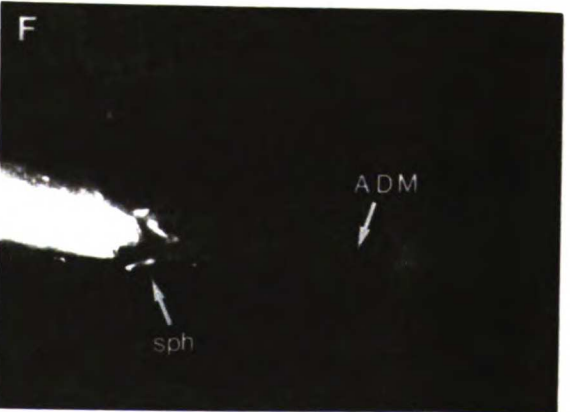
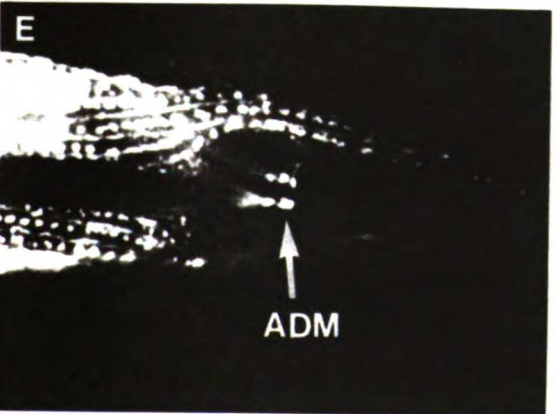
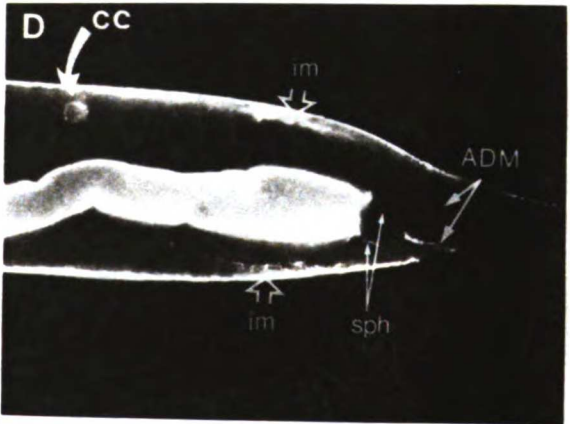
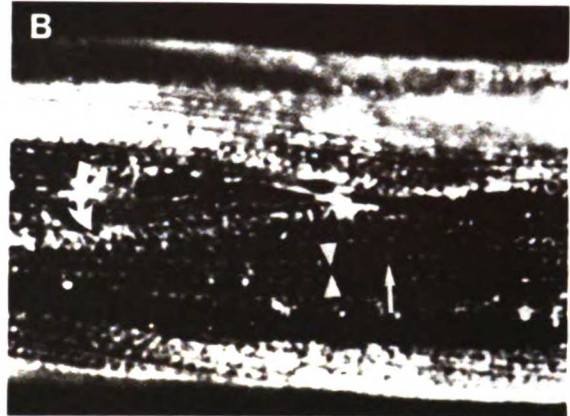
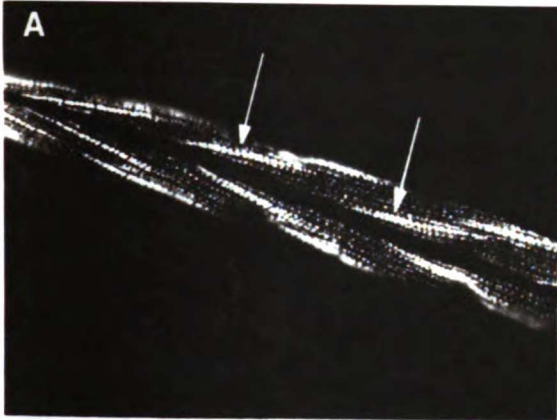
muscles, which open the vulva during egg-laying, *C. elegans* integrin β pat-3 was located ventrally, in apposition to the opening of the vulva, and laterally, along the uterus (Fig. 8G, H and K). *C. elegans* integrin β pat-3 was also detected in the uterine muscles localized to the lateral ridge (Fig. 8I). Interestingly, no staining was detected in the muscles comprising the pharynx (data not shown).

In addition to muscle cells, *C. elegans* integrin β pat-3 was detected in several other tissues. The somatic gonad, the uterus, the ovary, and the spermatheca were stained with the β pat-3 cytoplasmic antibody (Figs. 8G, I, and L). All six coelomocytes, which have gland-like morphologies and may act as scavenger cells, have *C. elegans* integrin β pat-3 localized on the surface of the cell in a uniformly punctate pattern (Figs. 8D, G K and L, Fig. 9F). Lastly, the apical surface of the intestine was strongly stained with the β pat-3 cytoplasmic antibody. As this region has been observed to bind other antibodies non-specifically (Ed Hedgecock, pers. comm.), *pat-3(rh54)* embryos, which should not express *C. elegans* integrin β pat-3 (see following chapter), were stained with the β pat-3 cytoplasmic antibody. Intestinal staining was observed in *pat-3(rh54)* embryos, suggesting that the intestinal staining is not dependent on the expression of *C. elegans* integrin β pat-3 (Fig. 8N). However, staining was not detected in muscles, in coelomocytes, or in the pharynx in these embryos, providing additional evidence that the staining observed in these tissues is due to *C. elegans* integrin β pat-3 expression.

C. elegans integrin β pat-3 is expressed during embryogenesis

Embryos were examined with the β pat-3 cytoplasmic antibody to determine when *C. elegans* integrin β pat-3 was expressed relative to the differentiation of the tissues in which it is present post-embryonically. At the comma stage of embryogenesis (approximately 400 minutes), weak staining in body wall muscle quadrants was detected (Fig. 9A). At this stage, the twitching movements which indicate functional muscles are first observed. Staining was also detected in the pharynx and tentatively assigned to the marginal cells, although further work will be necessary to verify this assignment (Fig. 9B). By the 1^{1/2} fold stage of embryogenesis (approximately 430 minutes), strong staining was detected in the body wall muscles (Fig. 9C) and in the pharynx (Fig. 9D). After the 3-fold stage of embryogenesis (approximately 520 minutes), staining was no longer detected in the pharynx (Fig. 9F). However, staining in the anal depressor muscle and the coelomocytes, which have migrated to their adult position by this stage, was present (Fig. 9E and F). In addition, transient labeling of several processes, which appeared to be

Figure 2-8. Immunocytochemical analysis of whole-mount worms using the β pat-3 cytoplasmic antibody. a) β pat-3 staining in two quadrants of body wall muscles, 63x objective. Arrows indicate cell boundaries. b) Enlarged view of β pat-3 staining in body wall muscle cells showing localization to the dense bodies (arrow) and M lines (arrowheads). c) β pat-3 staining in the diagonal muscles (DM) of the male tail at the lateral site of attachment to the body wall. d) Lateral view, showing β pat-3 staining of the ventral attachment sites (apposed to the anus) and the middle strut of the anal depressor muscle (ADM), the ventral attachment sites of the sphincter muscle (sph), the basal surface of the intestinal muscles (IM) and the cell surface of the left post-embryonically derived coelomocyte (cc). The fluorescence in the intestine is most likely not due to β pat-3 expression as it is observed in *pat-3(rh54)* embryos (see 8n). β pat-3 staining in the dorsal and ventral body wall muscle quadrants is out of the plane of focus. e) Ventral view, showing β pat-3 staining in the anal depressor muscle (ADM). f) Ventral view, same animal as in (e) but in a more dorsal plane of focus, revealing β pat-3 staining at the middle strut of the anal depressor muscle (ADM) and the ventral attachment site of the sphincter muscle (sph). g) Lateral view, left side, showing β pat-3 staining at the ventral and lateral attachment sites of the vm1 and vm2 vulval muscles, the anterior spermatheca (sp), and the embryonically-derived coelomocytes (cc). h) Ventral view of β pat-3 staining at the ventral attachment sites of the vm1 vulval muscles, which flank the opening of the vulva. i) Lateral view, right side, showing β pat-3 staining in the vulval muscles as described in (h), and staining at the lateral ridge attachment sites of the um1 uterine muscles and in the posterior portion of the uterus (Ut) of a young adult hermaphrodite. j) Nomarski image of the same animal as in (i), showing the vulva and the posterior portion of the uterus (Ut). k) Lateral view, left side, showing β pat-3 staining in the vulval muscles as described in (i); in addition, the ventral attachment sites of the vm2 vulval muscles are more clearly visible. Staining in the posterior spermatheca (sp) and the embryonically-derived coelomocytes (cc) can also be seen. l) Double exposure of the animal shown in (k) with DAPI to show sperm nuclei contained in the spermatheca (sp) and the small nuclei characteristic of coelomocytes (cc). m) Animals stained with the β pat-3 cytoplasmic antibody pre-incubated with 10 μ g/ml of the cytoplasmic peptide show no detectable staining. Animals were co-labeled with MH27 to ensure that the animals had been permeabilized. n) Staining of *pat-3(rh54)* embryos with the β pat-3 cytoplasmic antibody and MH27 show that staining in the intestine (int) is not dependent on the expression of β pat-3, whereas staining in muscles, the pharynx (in embryos, see Figure 9), and coelomocytes is dependent on β pat-3 expression.



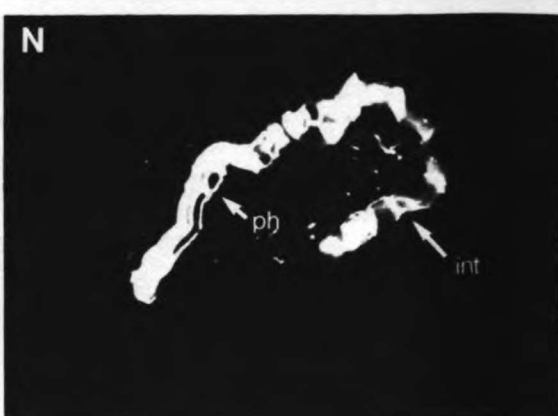
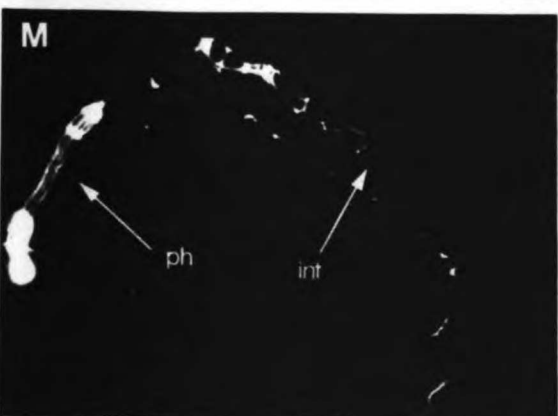
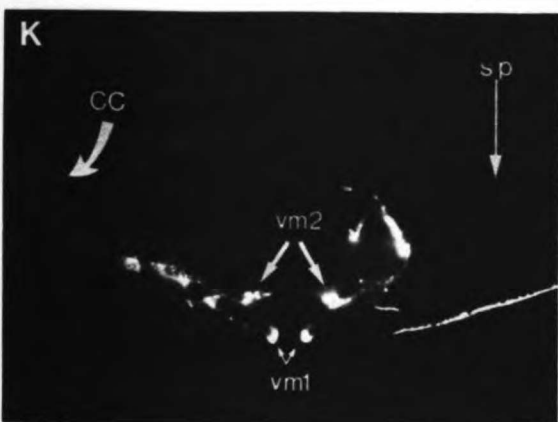
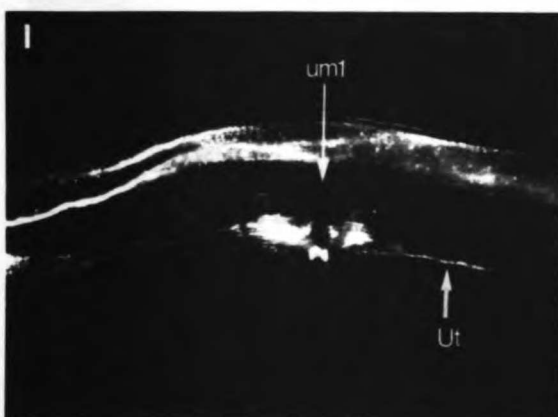
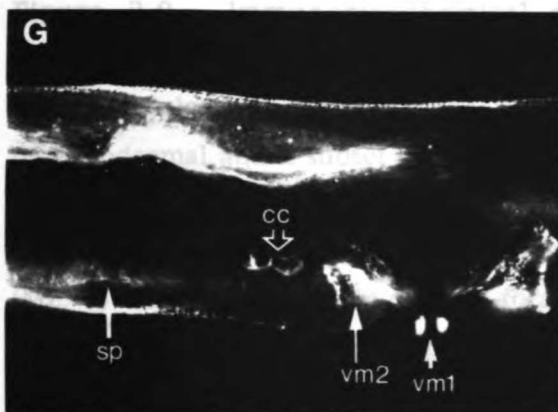
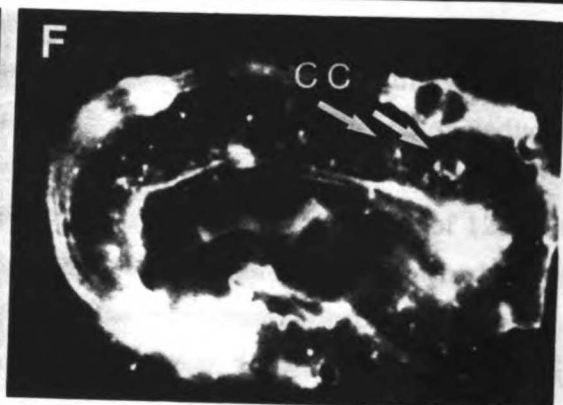
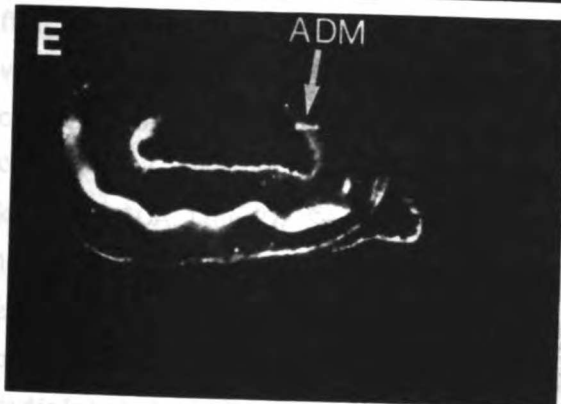
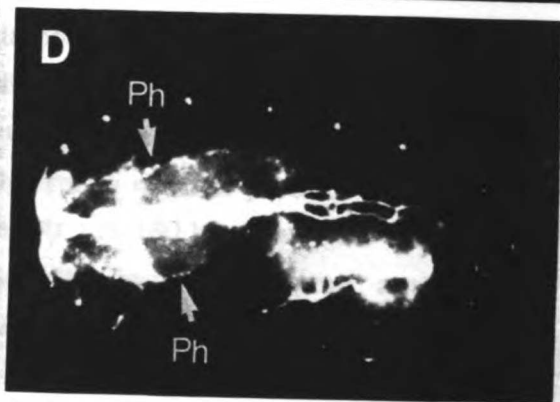
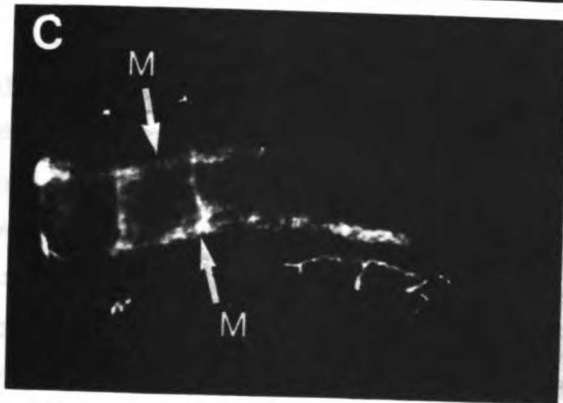
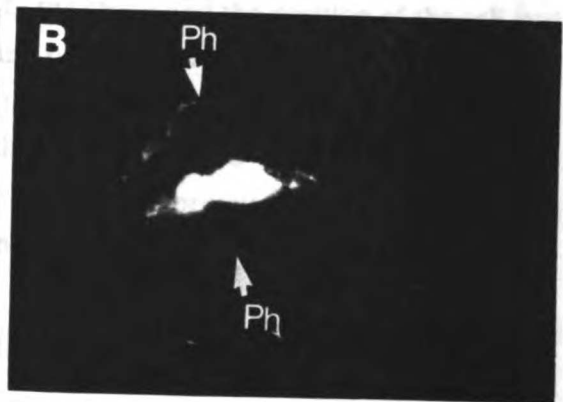
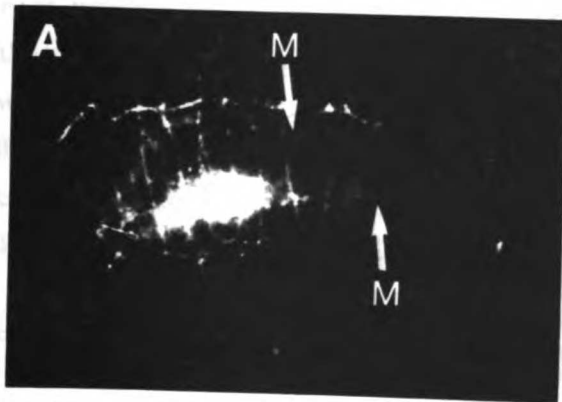


Figure 2-9. Immunocytochemical analysis of whole-mount embryos using the β pat-3 cytoplasmic antibody. All embryos except those shown in (e) and (g) were co-labeled with the monoclonal antibody MH27, which recognizes the apical surface of hypodermal and a subset of pharyngeal cells. The pattern of MH27 staining, which forms a grid-like pattern in hypodermal cells, can be seen in (h). a) Dorsal view of staining in a comma stage embryo, showing β pat-3 staining in developing muscle quadrants (M). b) Same embryo as in (a), mid-focal plane of embryo, showing β pat-3 staining in a subset of the pharynx (Ph), tentatively identified as the marginal cells. c) Dorsal view of a 1 $\frac{1}{2}$ - fold embryo, showing β pat-3 staining in the dorsal muscle quadrants (M). d) Same embryo as in (c), mid-focal plane of embryo, showing staining in a subset of the pharynx (Ph), again tentatively identified as the marginal cells. e) 3-fold embryo, showing β pat-3 staining in the anal depressor muscle (ADM). β pat-3 staining in body wall muscles is out of focus. Staining in the intestine (not marked) is most likely independent of β pat-3 expression as it was also detected in *pat-3(rh54)* embryos (see Fig. 8N) f) 3-fold embryo, right side, showing β pat-3 staining in the coelomocytes (cc). g) 3-fold embryo, showing β pat-3 staining in a neuronal cell body and process located laterally in the anterior portion of the embryo, with the process extending anteriorly. By the shape and position of the cell body (arrowhead), the direction and the timing with which this process is growing out (small arrow), this neuron is likely to be the touch neuron ALM.



neuronal, was observed. The neuron shown in Fig. 9G has been tentatively identified as ALM, which is one of the touch neurons, due to the shape and the position of the cell body, the direction of the projection, and the timing of the outgrowth of the process. Further work will be needed to unambiguously identify the processes which were stained by the β pat-3 cytoplasmic antibody. Finally, although many stages of development were examined, the analysis was not exhaustive; therefore expression of *C. elegans* integrin β pat-3 of a more transient nature, such as in migrating cells, may have been missed.

Putative integrin α subunits can be co-precipitated with C. elegans integrin β pat-3

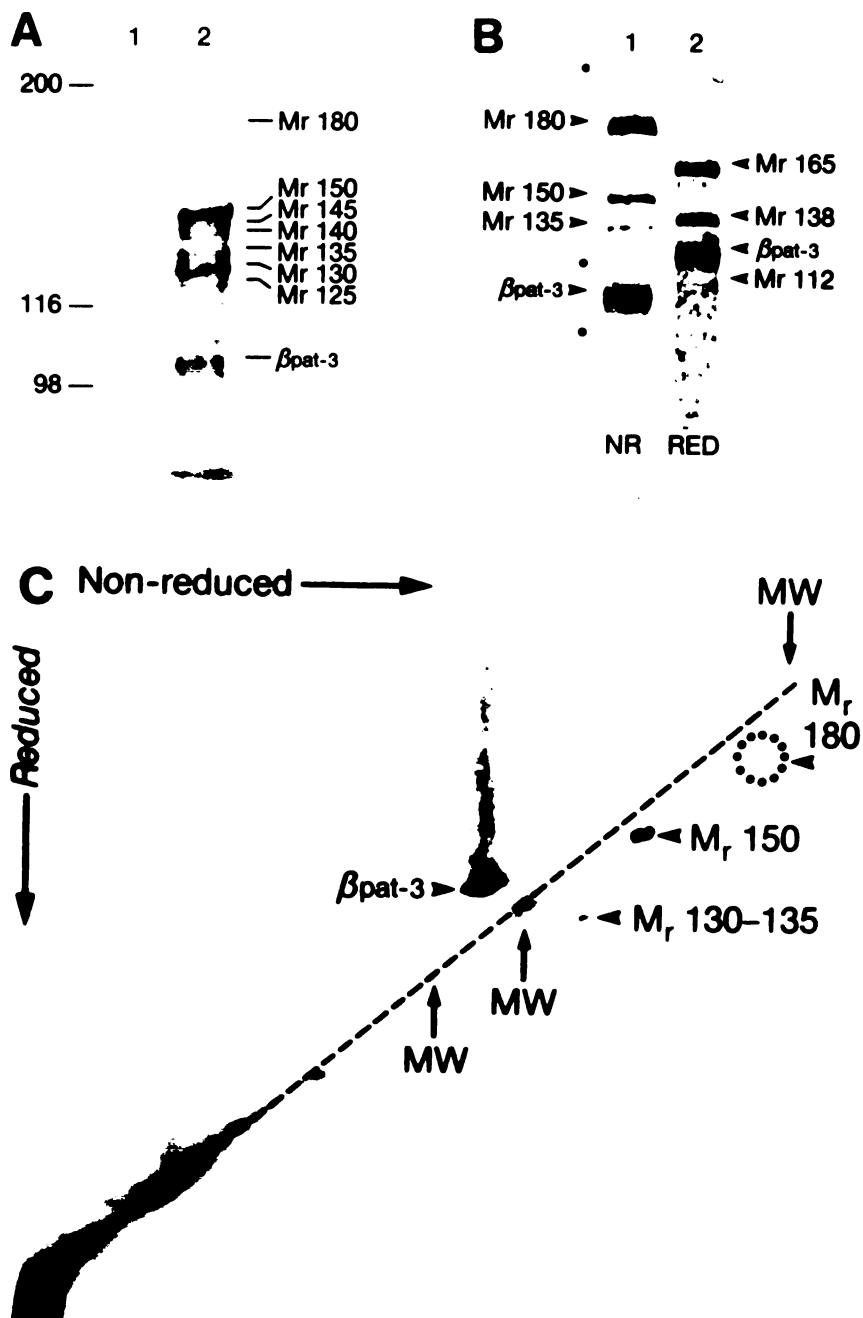
The observation that *C. elegans* integrin β pat-3 is present in several tissues and discretely localized in multiple structures within body wall muscle cells suggests that *C. elegans* integrin β pat-3 may associate with multiple integrin α subunits in order to carry out distinct functions within each cell. To determine whether proteins in the molecular weight range observed for integrin α subunits (Mr 125-180 kDa) associate with *C. elegans* integrin β pat-3, immunoprecipitations using the β pat-3 cytoplasmic antibody were performed. The conditions for the immunoprecipitations were identical to those used for vertebrate integrins, and two different methods for labeling protein were used. In the first, worm protein extracts were biotinylated and the immunoprecipitated proteins were visualized by the alkaline phosphatase reaction product or chemiluminescence. A chemiluminescence-derived autoradiogram is shown in Figure 10A. Under non-reducing conditions, *C. elegans* integrin β pat-3 is represented by a relatively broad band at Mr 109 kDa (Fig. 10A, lane 2). Seven additional protein bands with approximate Mr of 125 kDa, 130 kDa, 135 kDa, 140 kDa, 145 kDa, 150 kDa, and 180 kDa can be distinguished in this experiment. No bands in this molecular weight range were immunoprecipitated by the preimmune serum (Fig. 10A, lane 1). In the second method, worms were metabolically radiolabeled with ^{35}S and the immunoprecipitated proteins were visualized by autoradiography (Fig. 10B). Due to lack of sensitivity in this method, only a subset of the bands observed in the biotinylation method can be observed. Under non-reducing conditions (Fig. 10B, lane 1), *C. elegans* integrin β pat-3 is seen at approximately Mr 106 kDa, and three major bands with approximate Mr of 135 kDa, 150 kDa and 180 kDa are present. In addition, a minor band of Mr 140 kDa can be detected.

A majority of integrin α subunits are proteolytically cleaved into two disulfide-linked chains, and as a result, and their mobility in SDS-PAGE increases when electrophoresed under reducing conditions. To learn whether any of the proteins co-precipitated with

Figure 2-10. Immunoprecipitation of β pat-3 complexes using the β pat-3 cytoplasmic antibody. a) *C. elegans* N2 membrane proteins were labeled with sulfo-NHS-biotin and immunoprecipitated by the preimmune serum (lane 1) and the β pat-3 cytoplasmic antibody (lane 2). The immunoprecipitated proteins were electrophoresed under non-reducing conditions through a 6% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The blot was incubated with HRP-conjugated avidin and developed with reagents for chemiluminescence. The immunoprecipitated proteins were visualized by autoradiography. *C. elegans* integrin β pat-3 is represented by a band at 109 kDa (lane 2). Seven other bands with relative molecular weights between 125 kDa and 180 kDa can be detected. The numbers at the left indicate the positions and sizes of the molecular weight markers in Daltons $\times 10^3$.

b) Radiolabeled proteins were immunoprecipitated with the β pat-3 cytoplasmic antibody from a protein extract prepared from *C. elegans* N2 worms metabolically labeled with ^{35}S . The immunoprecipitated proteins were separated on a 6% SDS-polyacrylamide gel and visualized by autoradiography. Under non-reducing conditions (lane 1), *C. elegans* integrin β pat-3 and three major bands at Mr 130 kDa, 150 kDa, and 180 kDa can be detected. Under reducing conditions (lane 2), *C. elegans* β pat-3 and bands at Mr 112 kDa, 138 kDa and 165 kDa can be detected. The molecular weight markers are the same as in (a); their positions are indicated on the left by dots.

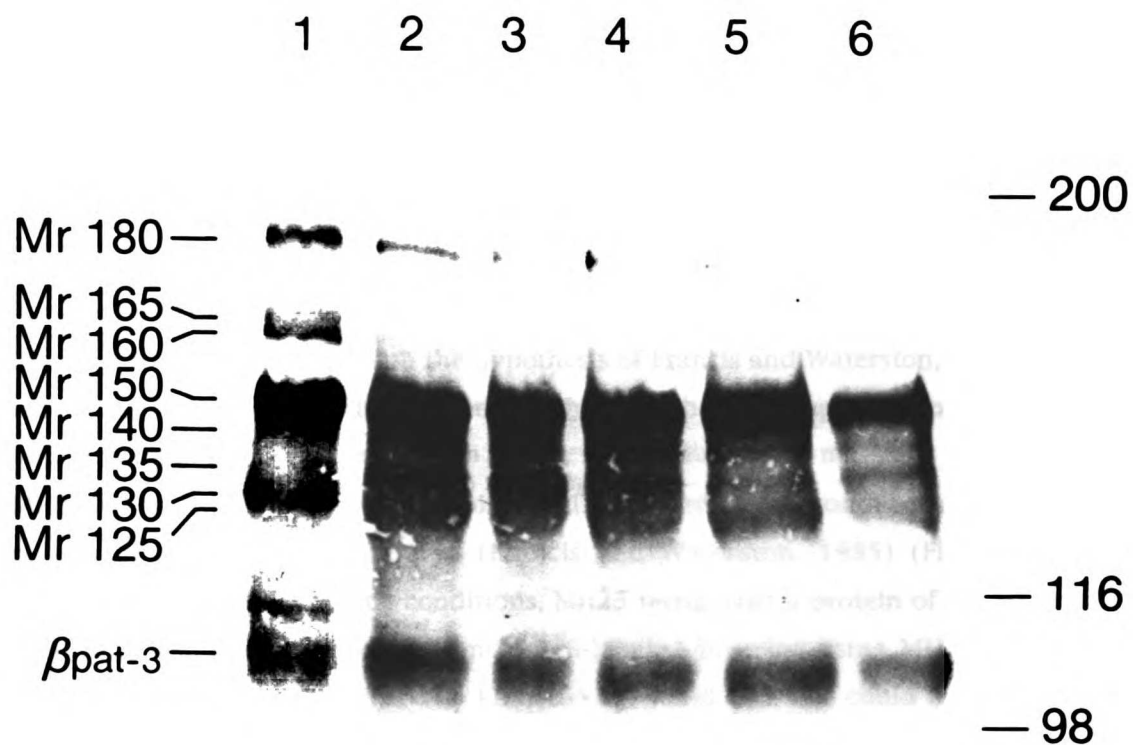
c) Two-dimensional SDS-PAGE of proteins immunoprecipitated with the β pat-3 cytoplasmic antibody. *C. elegans* N2 membrane proteins were labeled with sulfo-NHS-biotin and immunoprecipitated with the β pat-3 cytoplasmic antibody. The immunoprecipitated proteins were electrophoresed through a 6% SDS-polyacrylamide gel under non-reducing conditions in the first dimension and a 7.5% SDS-polyacrylamide gel under reducing conditions in the second dimension before transfer onto nitrocellulose. The nitrocellulose blot was incubated with alkaline phosphatase-conjugated streptavidin and developed with reagents for the alkaline phosphatase reaction. Pre-stained non-reduced molecular weight markers and biotinylated molecular weight markers pre-treated with iodoacetamide were included in the sample. *C. elegans* integrin β pat-3 migrates above the diagonal demarcated by the molecular weight markers, and proteins with approximate Mr of 135 kDa, 150 kDa, and 180 kDa in the first dimension migrate below the diagonal. The position of the 180 kDa protein is indicated by a dashed circle as it is faint. The molecular weight markers are the same as in (a); their positions are marked by arrows labeled MW.



C. elegans integrin β pat-3 migrate with increased mobility when reduced, ^{35}S -labeled immunoprecipitates were electrophoresed under reducing conditions (Fig. 10B, lane 2). *C. elegans* integrin β pat-3, as shown by immunoblot analysis (Fig. 7), migrates at Mr 120 kDa when reduced. Interestingly, the Mr 135, Mr 150 kDa and Mr 180 kDa bands observed under non-reducing conditions (Fig. 10B, lane 1) have also shifted; instead, bands with approximate Mr of 112 kDa, 138 kDa, and 165 kDa are observed (Fig. 10B, lane 2). To determine by precisely how much the bands have shifted, immunoprecipitates were electrophoresed in two dimensional SDS-PAGE. Proteins were electrophoresed under non-reducing conditions in the first dimension and under reducing conditions in the second (Fig. 10C). *C. elegans* integrin β pat-3, as expected, migrated above the diagonal demarcated by the molecular weight markers. In addition, several bands were detected below the diagonal: the Mr 180 kDa band migrated with an approximate Mr of 165 kDa when reduced (data not shown in this experiment), the Mr 150 kDa band migrated with an approximate Mr of 135 kDa, and a third band with an Mr of 130-135 kDa migrated at approximately Mr 112 kDa. The resolution of the two-dimensional gel was not sufficient to determine whether this third band represents one or more of the Mr 130 kDa, 135 kDa, and 140 kDa bands shown in Figure 10A, lane 2. The relative molecular weights of these co-precipitated proteins decreased by approximately 15-25 kDa when reduced, which is equivalent to the decrease in molecular weight seen when integrin α subunits known to be composed of two disulfide-linked chains are electrophoresed under reducing conditions (Hynes, 1987). Thus, at least three of the proteins co-precipitated with *C. elegans* integrin β pat-3 have a biochemical property in common with known integrin α subunits.

To determine whether the levels of proteins that co-precipitate with *C. elegans* integrin β pat-3 change during development, immunoprecipitations from six developmentally staged populations of worms were performed (Fig. 11): embryos (lane 1); young larvae (L1/L2, lane 2); larvae beginning to undergo extension of the sexual structures (L3/L4, lane 3); larvae in which the uterus is developing combined with young adults without oocytes (L4/young adults, lane 4); gravid adult hermaphrodites (lane 5); and dauer larvae (lane 6). The dauer stage is an alternative third larval stage induced by overcrowding and food limitation. For this experiment, the dauer-constitutive mutant *daf-2(e1370)* was used to ensure a pure population of dauers. A total of nine bands with distinct mobilities co-precipitating with *C. elegans* integrin β pat-3 could be distinguished. Seven of these bands were identical to those described above (Fig. 10A). Interestingly, two bands of Mr 160 kDa and 165 kDa appear to be specific to embryos (Fig. 11, lane 1). The band migrating above *C. elegans* integrin β pat-3 (Fig. 11, lane 1, not marked) has not

Figure 2-11. Immunoprecipitations with the β pat-3 cytoplasmic antibody from developmentally-staged populations of *C. elegans*. Proteins labeled with sulfo-NHS-biotin from six stages of development were immunoprecipitated using the β pat-3 cytoplasmic antibody, electrophoresed through a 6% SDS-polyacrylamide gel under non-reducing conditions and transferred onto nitrocellulose. The nitrocellulose blot was incubated with alkaline phosphatase-conjugated streptavidin and the immunoprecipitated proteins visualized by the alkaline phosphatase reaction product. The stages of development that were examined were embryos, lane 1; young larvae (L1/L2), lane 2; larvae beginning to undergo development of the sexual structures (L3/L4), lane 3; larvae in which the uterus is developing combined with young adults without oocytes (L4/young adults), lane 4; gravid adult hermaphrodites, lane 5; and dauers, (lane 6). Dauers were obtained for this experiment using the dauer-constitutive mutant strain *daf-2(e1370)*. Two embryonic-specific bands of Mr 160 and 165 kDa can be seen (lane 1); in addition, the Mr 180 kDa band cannot be detected in dauers (lane 6). The numbers at the left indicate the positions and sizes of the molecular weight markers in Daltons $\times 10^3$.



been observed reproducibly. The 125 kDa band co-precipitated with *C. elegans* integrin β pat-3 appears most clearly as a doublet with the 130 kDa band in gravid adults (Fig. 11, lane 5). Also of note, the Mr 135 kDa and Mr 140 bands are more highly expressed in larvae (Fig. 11, lanes 2, 3 and 4) than embryos, gravid adults, or dauers (Fig. 11, lanes 1, 5, and 6). The Mr 150 kDa band is highly labeled in these experiments, and the level of its expression relative to *C. elegans* integrin β pat-3 appears to remain constant post-embryonically. The Mr 145 kDa band is difficult to distinguish due to the strong labeling of the Mr 150 kDa band, and is not marked in Figure 11. Finally, the Mr 180 kDa band is present in all stages except for dauer. The properties of these bands are summarized in Table 1.

The monoclonal antibody MH25 recognizes C. elegans integrin β pat-3

The observation that the β pat-3 cytoplasmic antibody stains muscle cells in a pattern identical to MH25 together with the hypothesis of Francis and Waterston, 1985, that MH25 recognizes a transmembrane component of the dense body prompted us to examine whether MH25 recognizes *C. elegans* integrin β pat-3. On immunoblots, no bands were detected by MH25 when samples were electrophoresed under reducing conditions, confirming the results of Francis and Waterston (Francis and Waterston, 1985) (Fig. 12A, lane 1). However, under non-reducing conditions, MH25 recognized a protein of Mr 109 kDa (Fig. 12A, lane 4). Immunoprecipitation of ^{35}S -labeled proteins using MH25 revealed that bands with approximate Mr of 106 kDa, 135 kDa, and 150 kDa could be detected under non-reducing conditions (Fig. 12B). Bands of these sizes were also detected in parallel immunoprecipitations performed using the β pat-3 cytoplasmic antibody (see above, Fig. 10B, lane 1). Interestingly, the Mr 180 kDa band immunoprecipitated by the β pat-3 cytoplasmic antibody was not detected, suggesting that MH25 may recognize a subset of the complexes immunoprecipitated by the β pat-3 cytoplasmic antibody. To investigate this possibility, reciprocal immunodepletions of biotinylated protein extracts using MH25 and the β pat-3 cytoplasmic antibody were performed (Fig. 12C). As predicted, after four sequential immunodepletions of the protein extract with MH25, the β pat-3 cytoplasmic antibody immunoprecipitated a Mr 109 kDa band with associated bands of Mr 130 kDa and Mr 135 kDa, and an Mr 180 kDa band (not shown in this experiment). In addition, as observed for the immunoprecipitation of ^{35}S -labeled proteins, the 180 kDa band was not immunoprecipitated by MH25 when proteins were labeled with biotin (Fig. 12C, lanes 1 and 6). Unexpectedly, the β pat-3 cytoplasmic antibody did not deplete the protein extract of the Mr 109 kDa band or the co-precipitated proteins. After four sequential

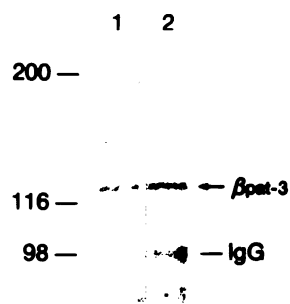
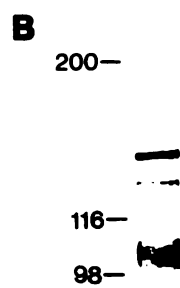
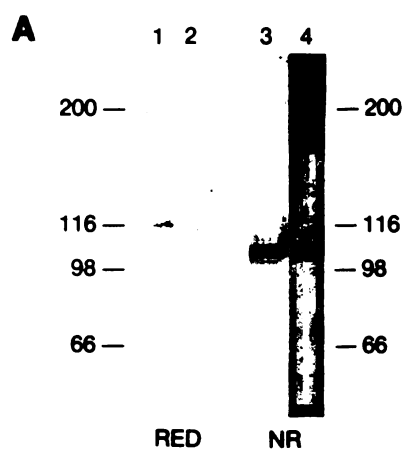
immunodepletions using the β pat-3 cytoplasmic antibody, MH25 immunoprecipitated bands with Mrs of 109 kDa, Mr 125 kDa, Mr 130 kDa, Mr 135 kDa, and 150 kDa. To ensure that MH25 recognizes β pat-3, non-labeled proteins were immunoprecipitated by MH25 and the β pat-3 cytoplasmic antibody, electrophoresed under reducing conditions, electroblotted onto nitrocellulose, and the blot incubated with the β pat-3 cytoplasmic antibody (Fig. 12D, lanes 1 and 2, respectively). A band of Mr 120 kDa can be detected in both lanes, confirming that MH25 recognizes β pat-3. Thus, the results of immunodepletions suggest that MH25 and the β pat-3 cytoplasmic antibody recognize overlapping subsets of co-precipitated proteins with the same molecular weights.

Figure 2-12. Immunoblot analysis and immunoprecipitations using the monoclonal antibody MH25. a) A protein extract was prepared from *C. elegans* N2 worms, separated on a 6% SDS-polyacrylamide gel under non-reducing conditions (lanes 1) and under reducing conditions (lanes 2), and electroblotted into a nitrocellulose membrane. The nitrocellulose was incubated with MH25. A band of Mr 109 kDa could be detected under non-reducing conditions but no bands were detected under reducing conditions. The numbers at the left indicate the positions and sizes of the molecular weight markers in Daltons x 10³ in all figures.

b) ³⁵S-labeled proteins from *C. elegans* N2 worms were immunoprecipitated using MH25, separated on a 6% SDS-polyacrylamide gel under non-reducing conditions and visualized by autoradiography. Major bands at Mr 109 kDa, 130 kDa, and 150 kDa can be detected. The Mr 180 kDa band immunoprecipitated by the β pat-3 cytoplasmic antibody was not detected.

c) Reciprocal immunodepletions using the β pat-3 cytoplasmic antibody and MH25. Biotinylated proteins were immunoprecipitated by four successive incubations with the β pat-3 cytoplasmic antibody (lanes 1-4) and MH25 (lanes 6-9) before a final immunoprecipitation with MH25 (lane 5) and the β pat-3 cytoplasmic antibody (lane 10), respectively. The samples were electrophoresed under non-reducing conditions. After the immunodepletion of the extract, MH25 can immunoprecipitate proteins with Mr of 109, 125, 130, 135, and 150 kDa, while the β pat-3 cytoplasmic antibody immunoprecipitates proteins with Mr of 109, 130, 135, and a 180 kDa band (180 kDa band not shown in this experiment) (lane 10).

d) MH25 and β pat-3 cytoplasmic antibody immunoprecipitates immunoblotted with the β pat-3 cytoplasmic antibody. Unlabeled proteins immunoprecipitated by MH25 (lane 1) and the β pat-3 cytoplasmic antibody (lane 2) were separated on a 6% SDS-polyacrylamide gel under reducing conditions and electroblotted onto nitrocellulose. The blot was incubated with the β pat-3 cytoplasmic antibody. Bands of Mr 120 kDa can be detected in both lanes.



DISCUSSION

As a first step toward understanding the function of integrin receptors during *C. elegans* development, we have cloned an integrin β subunit from *C. elegans* using the polymerase chain reaction and degenerate oligonucleotides designed from the highly conserved RGD-binding region of integrin β subunits. A genomic fragment containing the entire coding region of the *C. elegans* integrin β subunit was shown to rescue the embryonic lethality of the *C. elegans* mutation *pat-3(rh54)* in the following chapter; thus, the integrin β subunit described in this chapter has been named *C. elegans* integrin β pat-3. The pattern of expression of *C. elegans* integrin β pat-3 was determined by staining whole mount preparations of worms with an affinity-purified polyclonal antibody generated against a peptide designed from the predicted cytoplasmic domain of *C. elegans* integrin β pat-3. The immunocytochemical analysis revealed that *C. elegans* integrin β pat-3 was expressed in several tissues in the embryo and post-embryonically, with a particularly striking pattern of subcellular localization in body wall muscles. Immunoprecipitations using the β pat-3 cytoplasmic antibody showed that at least nine distinct protein bands with molecular weights in the range observed for integrin α subunits co-precipitate with *C. elegans* integrin β pat-3. A subset of these bands were also immunoprecipitated by the monoclonal antibody MH25, which was shown in this study to recognize *C. elegans* integrin β pat-3.

Three lines of evidence suggest that we have determined the complete sequence of the *C. elegans* integrin β pat-3 transcript. First, the longest cDNA isolated (cDNA 1-1) was equivalent in length to the major transcript observed by RNA blot analysis. Second, 11 nt of an SL1 variant *trans*-splice leader sequence was present at the 5' end of the longest cDNA isolated, and a poly A stretch was present at the 3' end. Third, as a single-length transcript was detected and no potential exons were found in intron sequences, it is unlikely that alternatively-spliced transcripts containing exons not present in cDNA 1-1 exist. However, since the genomic DNA was only sequenced 500 bases 3' to the last exon containing the carboxy-terminal end of the cytoplasmic domain, the possibility that this exon is alternatively-spliced has not been ruled out. This exon has been observed to be alternatively-spliced in the human integrin β 1 and β 3 subunits (van Kuppevelt et al., 1989; Altruda et al., 1990).

Analysis of the *C. elegans* integrin β pat-3 gene revealed that the coding sequence spans approximately 5.2 kB. A 9 kB Sal-1 fragment containing the entire coding region of the

gene and approximately 1.5 kB of sequence 5' to the proposed initiator methionine was sufficient to rescue the embryonic lethal phenotype of the *pat-3(rh54)* mutation, as well as several post-embryonic phenotypes examined in the rescued animals (see following chapter). This result suggests that the coding sequence and necessary regulatory elements of the *C. elegans* integrin β pat-3 gene are contained within 9 kB of genomic DNA. Sequence analysis of the 9 kB Sal-1 fragment showed that the organization of the *C. elegans* integrin β pat-3 gene is less complex than that of the human integrin β 2, β 3 and β 7 genes, with approximately half the number of introns as the human genes. The length of the introns in the *C. elegans* integrin β pat-3 gene were relatively short, consistent with what has been observed for other *C. elegans* introns (Wood, 1988) although none were 48 or 52 bases, a length often observed. Of the seven *C. elegans* integrin β pat-3 introns, four had counterparts in the same location in the human genes, and two of these four were in the same location in the *Drosophila* integrin β PS gene. Like the human integrin β 3, β 5, and β 6 genes, and the *Drosophila* integrin β PS gene, the amino acid sequence corresponding to the ligand binding region of *C. elegans* integrin β pat-3 is contained within a single exon. In contrast, the human integrin β 1, β 2, and β 7 genes contain an intron in this region, dividing it into two exons (Jiang *et al.*, 1992). Interestingly, the four cysteine-rich repeats characteristic of integrin β subunits were not encoded by discrete exons in any of the genes.

The pattern of *C. elegans* integrin β pat-3 expression was examined to gain insight into the function of *C. elegans* integrin β pat-3 during development. The most prominent staining of *C. elegans* integrin β pat-3 was in muscle cells, where *C. elegans* integrin β pat-3 was localized to points of cell attachment. In body wall muscle, *C. elegans* integrin β pat-3 was concentrated in the dense bodies, the M lines, and the obliquely oriented borders between muscle cells. The borders between cells contain attachment plaques; further work is necessary to determine whether *C. elegans* integrin β pat-3 is a component of these plaques. The pattern of staining in body wall muscles with the β pat-3 cytoplasmic antibody was identical to that seen with MH25 (Francis and Waterston, 1985). The hypothesis of Francis and Waterston (Francis and Waterston, 1985) that MH25 binds to a membrane or cell surface component of the dense body was shown to be correct in this study by the finding that MH25 recognizes *C. elegans* integrin β pat-3. Vinculin and α -actinin are also present in the dense bodies but not in the M lines, and α -actinin is absent from the attachment sites between muscle cells (Francis and Waterston, 1985). Thus, it is likely that as hypothesized in vertebrates (Tamkun *et al.*, 1986), *C. elegans* integrin β pat-3 provides a transmembrane link in some of these structures to the actin cytoskeleton via

vinculin and α -actinin. A diagram of a sarcomeric unit incorporating a model of the composition of the dense body from Francis and Waterston, (Francis and Waterston, 1985) is shown in Figure 13.

The pattern of *C. elegans* integrin β pat-3 localization in body wall muscles is similar to the pattern of integrin localization in vertebrate and *Drosophila* striated muscle, but not identical. As in *C. elegans*, the vertebrate integrin β 1 and *Drosophila* integrin β PS have been shown to localize in the Z bands (Bozyczko et al., 1989; Volk et al., 1990); however, these integrin β subunits are absent from the M lines. Moreover, while the vertebrate integrin β 1 and *Drosophila* integrin β PS are strongly localized at myotendinous junctions (Bao et al., 1993; Leptin et al., 1989), no increased density of staining can be detected at the distal ends of the body wall muscle cells, which would be the analogous site in *C. elegans*. Analysis of *C. elegans* *pat-3(rh54)* mutant muscles, which do not contain functional β pat-3 (see following chapter), confirmed that *C. elegans* integrin β pat-3-containing heterodimers do not function in the attachment of the distal ends of the cells to the basement membrane.

In contrast to *Drosophila* integrin β PS, where the integrin is present in ectodermal cells in embryos (Leptin, 1989), we did not detect *C. elegans* integrin β pat-3 in hypodermal cells, either embryonically or post-embryonically. Results presented in the accompanying study are consistent with no detectable function for the integrin in hypodermal cells. Moreover, unlike the vertebrate integrin β 1, which is highly expressed in the embryonic nervous system (reviewed in Reichardt and Tomaselli, 1991), no expression of *C. elegans* integrin β pat-3 was detected in the dorsal or ventral nerve cords, the nerve ring or the pre-anal ganglion. Consistent with this observation, results from the accompanying study have not revealed a role for *C. elegans* integrin β pat-3 in these neurons. However, transient staining was observed in a neuronal cell body and process tentatively identified as ALM, which was shown in the following chapter to require *C. elegans* integrin β pat-3 for its migration.

Biochemical analyses using the β pat-3 cytoplasmic antibody demonstrated that *C. elegans* integrin β pat-3 has features observed for previously isolated integrin β subunits. For example, under reducing conditions, *C. elegans* integrin β pat-3 migrated with increased mobility in SDS-PAGE as compared with non-reducing conditions. In addition, at least nine protein bands with molecular weights in the range observed for integrin α subunits co-precipitate with *C. elegans* integrin β pat-3. We believe that at least some and

possibly all of these bands may represent integrin α subunits for the following reasons. First, the conditions used to immunoprecipitate *C. elegans* integrin β pat-3 in this study were identical to those used for vertebrate integrins, and under these conditions, protein bands in the molecular weight range 120 kDa - 180 kDa co-precipitating with integrin β subunits have been shown to be integrin α subunits. Second, when the immunoprecipitates were electrophoresed in SDS-polyacrylamide gels under reducing conditions, at least three of the co-precipitated bands migrated with increased mobility. This result is significant because approximately 60% of identified integrin α subunits are proteolytically cleaved into a disulfide-linked heavy chain and light chain and, when reduced, migrate with increased mobility in SDS-PAGE (Hynes, 1987). Third, the level of expression of several of the co-precipitated bands appeared to change with development; most notably, two bands were detected only in embryos, and a third band was absent in dauer larvae. Fourth, bands with molecular weights immunoprecipitated by the β pat-3 cytoplasmic antibody were also immunoprecipitated by MH25, thereby demonstrating that these proteins are not non-specifically immunoprecipitated by the β pat-3 cytoplasmic antibody. Fifth, the *pat-3(rh151)* mutation has been shown to affect the association of these co-precipitated proteins with *C. elegans* integrin β pat-3 (see next chapter). Finally, an integrin α subunit has been cloned in the *C. elegans* genome sequencing project (Alan Coulson, pers. comm.), providing direct evidence that *C. elegans* has at least one integrin α subunit. Further work is needed to determine whether each of the bands co-precipitated with *C. elegans* integrin β pat-3 is an integrin α subunit and whether the integrin α subunit that has been cloned associates with *C. elegans* integrin β pat-3.

Results from immunoblot analysis and immunoprecipitations with MH25 have led us to conclude that MH25 recognizes *C. elegans* integrin β pat-3. However, our observations suggest that MH25 recognizes an epitope distinct from the epitope(s) recognized by the β pat-3 cytoplasmic antibody. For example, in contrast to the β pat-3 cytoplasmic antibody, MH25 does not recognize *C. elegans* integrin β pat-3 under reducing conditions on immunoblots. This result suggests that the epitope recognized by MH25 is dependent on disulfide linkages and therefore is most likely located in the extracellular domain. Furthermore, the Mr 180 kDa band co-precipitated with *C. elegans* integrin β pat-3 by the β pat-3 cytoplasmic antibody was not immunoprecipitated by MH25. One possible explanation is that the Mr 180 kDa band represents an integrin α subunit that when associated with *C. elegans* integrin β pat-3, masks or changes the conformation of the epitope recognized by MH25. In support of this hypothesis, sequences in the extracellular domain of integrins have been shown to be responsible for heterodimer

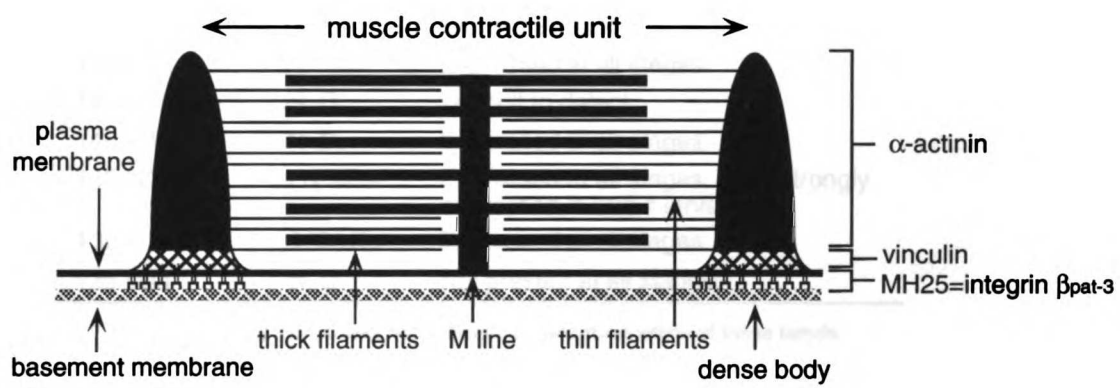
association (Bodary, et al., 1991; Dana et al., 1991; Wippler et al., 1994) and heterodimers can undergo conformational changes that reveal new epitopes in response to ligand binding and activation of second messengers (Coller, 1985; Shattil, et al., 1985; Altieri and Edgington, 1988; Gulino et al., 1990; O'Toole et al., 1991; Andrieux et al., 1991). The strongest argument that MH25 and the β pat-3 cytoplasmic antibody recognize different epitopes was provided by reciprocal immunoprecipitations experiments. Surprisingly, neither antibody completely depleted protein extracts of *C. elegans* integrin β pat-3 or the co-precipitated proteins. Whether the antibodies distinguish a functional difference in receptor structure remains to be determined.

One question raised by this study is whether *C. elegans* integrin β pat-3 is a homolog of the vertebrate integrin β 1 subunit, or whether it is representative of a prototypic integrin β subunit that existed before the evolutionary divergence of protostomes from deuterostomes. Several arguments favor the latter hypothesis. (1) Computer alignments revealed that *C. elegans* integrin β pat-3 was slightly more homologous with human integrin β 1 (41%) than with other integrin β subunits; however, since the level of identity between integrin β subunits found in the same species ranges from 28-55%, *C. elegans* integrin β pat-3 cannot be called a homolog of integrin β 1 on the basis of sequence homology. (2) Although *C. elegans* integrin β pat-3 was shown to have the potential to associate with multiple putative integrin α subunits, this property is not unique to integrin β 1: integrins β 2, β 3 and β 7 also associate with multiple integrin α subunits (Hynes, 1992). (3) Comparison of the localization of *C. elegans* integrin β pat-3 with chicken integrin β 1 in post-hatch muscle (discussed above) showed that their distribution is overlapping but not identical. Future studies will show whether the functions of other integrin β subunits present in vertebrate muscle are analogous to those performed by *C. elegans* integrin β pat-3; likewise, an integrin β subunit distinct from *C. elegans* integrin β pat-3 may function to attach the distal ends of muscle cells to the basement membrane. (5) Unlike integrin β 1, *C. elegans* integrin β pat-3 is not strongly expressed in the embryonic nervous system, nor was it shown in the *pat-3* mutants (see accompanying study) to play a major role in the development of the *C. elegans* nervous system. (6) The human integrin β 1 gene, like the human integrin β 2 and β 7 genes, contains an intron in the highly conserved RGD-binding region, while the human integrin β 3, β 5, and β 6 and *C. elegans* integrin β pat-3 genes do not. Most likely, this intron appeared after the divergence of nematodes from vertebrates but before the duplication and evolution of distinct vertebrate integrin β subunit genes.

The following chapter shows that integrin function can be studied in *C. elegans* with

single cell resolution. With the completion of the *C. elegans* genome sequencing project, it will soon be clear exactly how many integrin α and β subunits genes are present in *C. elegans*. Furthermore, as the genetic and physical maps are becoming increasingly linked and more mutants are isolated, the identification of mutations in other integrin genes will be facilitated. In particular, the identification of mutations in integrin α subunit genes whose protein products associate with *C. elegans* integrin β pat-3 should be informative, as their phenotypes are likely to be discrete. Using *C. elegans* genetics, it may then be possible to determine which proteins interact with integrin receptors to produce cellular behaviors such as cell motility and neurite outgrowth.

Figure 2-13. Model of the organization of the *C. elegans* body wall muscle sarcomere. The dense bodies and M lines are thought to provide the structural attachment sites for the thin and thick filaments, respectively. α -actinin (Francis and Waterston, 1985) and vinculin (Barstead and Waterston, 1989) have been shown to be components of the dense bodies. Francis and Waterston proposed that the antigen recognized by the monoclonal antibody MH25 is a membrane-bound or membrane-associated component of the dense body (Francis and Waterston, 1985). This hypothesis was shown to be correct in this study by the demonstration that MH25 recognizes *C. elegans* β pat-3.



INTEGRIN

Table 1. Summary of proteins co-precipitated with *C.elegans* integrin β_{pat-3} .

Mr (non-reduced)	Mr (reduced)	Comments
180 kDa	155 kDa	Not expressed in dauers or recognized by MH25.
165 kDa	N. D.	Embryo specific.
160 kDa	N. D.	Embryo specific.
150 kDa	138 kDa	Expressed at all stages.
145 kDa	N. D.	Difficult to detect.
140 kDa	N. D.	Expressed at all stages.
135 kDa	112 kDa*	Expressed at all stages; most strongly expressed in L3/L4 larvae.
130 kDa	112 kDa*	Expressed at all stages.
125 kDa	N. D.	Not present at all stages.

* The resolution of the gel was not sufficient to determine which of these bands, or whether both of these bands, have an increased mobility when reduced.

CHAPTER THREE

FUNCTIONAL ANALYSIS OF THE *C. ELEGANS* MUTANT *pat-3*, A LOCUS ENCODING A NOVEL INTEGRIN β SUBUNIT

PREFACE

The work presented in the following chapter is the result of a collaboration between Dr. Edward Hedgecock, Dr. Robert Herman, Dr. David Hall, Dr. John Plenefisch, Dr. Matthew Buechner and myself. Dr. Hedgecock has kindly provided me with a preliminary version of our manuscript, which formed the basis of the following chapter.

Dr. Hedgecock and Dr. Herman isolated the *pat-3(rh54)* mutation in a screen for embryonic lethal mutations. The characterization of the embryonic lethal phenotype and determination of the post-embryonic phenotypes of *pat-3(rh54)* by mosaic analysis is their work. The electron microscope analysis of *pat-3(rh54)* phenotypes was carried out by Dr. Hall. Dr. Plenefisch initiated the collaboration by noting that the genetic location of *pat-3(rh54)* was near the physical location of the β integrin gene and contacting me with this information. In addition to *pat-3(rh54)*, Dr. Hedgecock isolated and characterized the phenotypes of the *pat-3(rh96)* and *pat-3(rh151)* mutations. Dr. Plenefisch and Dr. Buechner performed the complementation tests that showed that the *rh151* and *rh96* mutations, respectively, are allelic to *pat-3(rh54)*. Dr. Plenefisch found that the *pat-3(rh151)* phenotypes are not sensitive to gene dosage by examining one copy of the *pat-3(rh151)* mutation in *trans* to a deficiency. Dr. Buechner found that one of the *pat-3(rh96)* phenotypes is sensitive to gene dosage by examining one copy of the *pat-3(rh96)* mutation in *trans* to a deficiency.

My contributions to the collaboration include injecting animals containing the *pat-3(rh54)* mutation with the integrin β subunit gene and observing the rescue of the embryonic lethal phenotype, determining the molecular nature of the mutations in the three *pat-3* alleles, and examining *pat-3(rh54)* using the β *pat-3* cytoplasmic antibody. In addition, I initiated a series of genetic experiments in Dr. Hedgecock's laboratory. I extended the findings of Dr. Plenefisch and Dr. Buechner by examining the effects of gene dosage on the *pat-3(rh96)* and *pat-3(rh151)* phenotypes by constructing strains containing the *pat-3(rh96)* and *pat-3(rh151)* mutations in *trans* to the *pat-3(rh54)* mutation and a strain containing two copies of the *pat-3(rh96)* mutation and one wild type copy of the gene. I also examined the neural migrations defects in *pat-3(rh54)* mosaic animals and in the two viable *pat-3* alleles, constructed *him-5(e1490)* strains of each allele and examined the phenotypes of the *pat-3(rh96)* and *pat-3(rh151)* mutations in males, and constructed and analyzed the phenotypes of *pat-3;unc-52* double mutants. Observations from this work that were not fully investigated are summarized in Appendix II.

SUMMARY

The *C. elegans* integrin β subunit gene cloned in the previous chapter maps to the left arm of chromosome III (A. Coulson, pers. comm.). Of the mutants that map to this region, *pat-3* (paralyzed, arrested elongation at two-fold) was a strong candidate for an integrin mutant because it has phenotypes consistent with known integrin function. These phenotypes include failure of the embryo to elongate and hatch as a viable larva, and post-embryonic defects, as shown by mosaic analysis, in muscle cell attachment, muscle sarcomere organization, somatic gonad and distal tip cell epithelialization, excretory cell process outgrowth, PLM axon outgrowth, and neural and non-neural cell migrations. A 9 kb Sal-1 fragment containing the integrin β subunit gene described in the previous chapter was injected into *pat-3(rh54)* and found sufficient to rescue the embryonic lethality and the post-embryonic phenotypes of this allele. Thus, the integrin β subunit cloned in the previous chapter has been named *C. elegans* integrin β pat-3. The molecular identities of the mutation in *pat-3(rh54)* and in two viable *pat-3* alleles were determined. *Pat-3(rh54)* contains a nonsense mutation, resulting in a truncation of the protein within the first 200 amino acids of the protein. Genetic evidence suggests that one of the viable alleles, *pat-3(rh96)* is an antimorph; that is, the *rh96* mutant integrin can interfere with wild type integrin function and produce phenotypes not observed in the null. Sequence analysis revealed that there is a conversion of R²⁷⁹ $\rightarrow$ C²⁷⁹, resulting in an additional cysteine near the putative ligand binding region. The second viable allele, *pat-3(rh151)*, appears to have tissue-specific lack of integrin function in a subset of the cells shown to require the integrin in *pat-3(rh54)*. The mutation in *pat-3(rh151)* results in the conversion of p²⁰³ $\rightarrow$ L²⁰³; this proline is located in the putative ligand binding region and is conserved in all integrin β subunits sequenced to date. Immunoprecipitations from *pat-3(rh151)* animals revealed that this mutation appears to affect the association of proteins which co-precipitate with *C. elegans* integrin β pat-3. Finally, *pat-3(rh151)*, but not *pat-3(rh96)*, appears to interact genetically in double mutant combinations with two viable alleles of *unc-52*. *unc-52* encodes the basal lamina heparin sulfate proteoglycan perlecan; thus, this result suggests that Unc-52 may be a ligand of receptors containing *C. elegans* integrin β pat-3.

INTRODUCTION

Understanding the contributions of integrin receptors to the development of an animal is a challenging problem: Perturbation experiments using function-blocking antibodies or anti-sense oligonucleotides may not allow a comprehensive survey of all tissues in which the receptor is expressed at each stage of development. Classical genetics can provide an alternative method for examining the role of integrins in development. With null mutations, complete lack-of-function phenotypes can be identified, and each subunit or subunit isoform can be individually mutated and the functional consequences of the mutations studied in living animals. Using site-directed mutations or chimeric genes, the roles of particular structural features can be analyzed by injection of constructs encoding the recombinant molecules into null mutants. Functional interactions between integrins, extracellular matrix molecules, and cytoskeletal proteins can be tested using double mutant combinations. Even unknown genes, whose products interact with specific integrins, can be identified by genetic methods such as suppressor analysis.

Certain aspects of integrin biology might appear to confound a genetic approach. First, many integrins are expressed at several stages of development, and abnormalities of early development may complicate or preclude observations of later stage phenotypes. Second, individual integrin receptors can be expressed in multiple tissues; therefore, autonomous cellular phenotypes caused by integrin loss of function can be compounded by non-autonomous effects due to altered integrin expression in neighboring cells. These first two obstacles can be largely overcome by the technique of mosaic analysis (Herman, 1989; Zusman et al., 1990). Third, integrins assemble and function as heterodimers, and each subunit may associate with more than one of the complementary subunits. Thus, null mutations in a particular subunit would disrupt all heterodimer combinations in which that subunit participates, both within the same cell and in different cells (Kishimoto et al., 1987b; Leptin et al., 1989). For studying the function of a single heterodimer pair, this may seem a serious limitation. However, the roles of distinct subunit combinations can still be inferred by comparing the phenotypes of each subunit mutated alone. Finally, integrins may have functions that overlap with other adhesion receptors. In this case, double mutants can reveal redundant gene functions not apparent in either single mutant alone.

The nematode *Caenorhabditis elegans* has several advantages as a genetic model for studying the role of integrins in animal development. The cellular anatomy of adult

hermaphrodites and males, which is simple and invariant, has been reconstructed from serial electron micrographs (Albertson and Thomson, 1976; White et al., 1976; White et al., 1986; Hall and Russell, 1991). These ultrastructural studies have also determined axon projections and patterns of synaptic connections. The animals, which are small and transparent, develop rapidly. Thus, cell lineages, morphogenetic movements and cell migrations can be traced in living embryos and larvae by differential interference contrast (DIC or Nomarski) microscopy (Sulston, 1976). Finally, the majority of the *C. elegans* genome has been cloned into an overlapping array of cosmids and yeast artificial chromosomes (YACs) (Coulson et al., 1986), and due to the cloning of genes for genetically isolated mutations, the physical map and the genetic map have been linked. Thus, it is possible to identify candidate mutants located near *C. elegans* genes cloned by homology to genes cloned in other organisms.

In this study, we report that the *pat-3* locus contains mutations in the coding sequence of the *C. elegans* integrin β subunit gene described in the previous study. For this reason, this integrin has been named *C. elegans* integrin β pat-3. The cellular phenotypes of two viable alleles, *pat-3(rh96)* and *pat-3(rh151)*, and the embryonic lethal allele *pat-3(rh54)* were determined by Nomarski microscopy. Post-embryonic phenotypes of the embryonic lethal allele *pat-3(rh54)* were determined by analyzing mosaic animals produced by the somatic loss of the free duplication, *sDp3*, which contains a wild type copy of the *C. elegans* integrin β pat-3 gene. The cellular phenotypes of *pat-3(rh54)* mosaic animals were characterized using a combination of light and electron microscopic methods. From these observations, we inferred specific roles for *C. elegans* integrin β pat-3 in muscle cell attachment and the organization of cytoarchitecture in multi-sarcomeric muscles cytoarchitecture, cytomorphogenesis, process outgrowth, and neuronal and non-neuronal cell migrations. Finally, additional biochemical and genetic experiments were carried out to examine the nature of the mutations in the two viable alleles.

MATERIALS AND METHODS

Animals

Reference alleles used in this study were: *dpy-1(e1)*, *dpy-17(e164)*, *dpy-27(y57)*, *ncl-1(e1865)*, *unc-36(e251)*, *unc-52(e669, e998, e1012, and e1421)*, *unc-79(e1068)*, *unc-93(e1500)*. Animals were maintained at 20°C as described in Wood, 1988. All genetic crosses were performed using standard genetic methods.

Rescue of the pat-3(rh54) mutation with the C. elegans integrin β subunit gene

The *C. elegans* integrin β subunit gene was mapped on linkage group III by fingerprint analysis (Alan Coulson, pers. comm.). The cosmid ZK1058 appeared to overlap the location of the integrin gene; Southern blot analysis of the cosmid with oligos β 135-PE and 7rev verified that it contained the entire coding sequence of the integrin gene. Initial injections with ZK1058 into the germlasm of *sDp3;dpy-1pat-3(rh54)ncl-1* animals were made with *rol-6* DNA as a dominant co-injection marker. As *dpy-1* is a recessive mutation covered by *sDp3*, the presence of F1 Dpy animals would indicate rescue of the *pat-3(rh54)* embryonic lethality. F1 Dpy animals were obtained from injection of ZK1058 DNA with and without *rol-6* DNA, and from injection of a 9 kb Sal-1 fragment containing the entire coding sequence of the *C. elegans* integrin β gene. Dpy animals were checked by Nomarski for the Ncl phenotype to ensure that they did not arise from a recombination event with *sDp3*. F1 Dpy animals were obtained from virtually every animal deemed to have been successfully injected.

Molecular characterization of the pat-3 mutations

The approximate locations of the *pat-3(rh54)*, *pat-3(rh96)* and *pat-3(rh151)* mutations in the *C. elegans* integrin β pat-3 gene were determined using the hydroxylamine procedure (Cotton et al., 1988), which modifies and cleaves DNA containing mismatched cytosines. Genomic DNA from the three *pat-3* mutants [*sDp3;dpy-1pat-3(rh54)ncl-1*, *pat-3(rh96)*, and *sDp3;pat-3(rh151)ncl-1*] and *C. elegans* strain N2 was prepared and amplified using the polymerase chain reaction (PCR) and an overlapping series of primers designed from the cDNA sequence to produce DNA fragments of 0.65 kb to 1.6 kb in length. The amplified DNA fragments were gel-purified using a gene-clean kit (Bio101, La Jolla, CA), and 10 ng of DNA from each PCR fragment was radiolabeled with 10 μ ci γ -³²P

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ATP (Amersham) using T4 polynucleotide kinase (Boehringer/Mannheim). After heat inactivation of the kinase at 65°C for 15 min, labeled DNA (*) was mixed with a tenfold excess of unlabeled DNA in 1x annealing buffer (0.3 M NaCl, 0.1 M Tris-Cl pH 8.0) in the following combinations: wild type*/wild type, wildtype*/mutant, wild type/mutant*, mutant/mutant*. The DNA mixtures were denatured at 100°C for 10 min and annealed at 65°C overnight. Following ethanol precipitation, the DNA was resuspended in freshly prepared 4 M hydroxylamine (Aldrich), 15% diethylamine (Aldrich), pH 6.0 and incubated at 37°C for 2 hr. Subsequently, the DNA was ethanol precipitated, resuspended in 1M piperidine (Aldrich), and incubated at 90°C for 30 min. Following a final precipitation with ethanol, the DNA was resuspended in sample buffer, boiled for 3 min, and loaded on a 6% polyacrylamide gel. The autoradiograph of the gel was examined for novel DNA fragments absent from control lanes. All three mutations were localized to a 400 bp region in the gene flanked by the oligonucleotide primers 1 and 2 rev (see previous chapter, Figure 4). DNA from this region was amplified by PCR from mutant genomic DNA using these primers, subcloned into Bluescript II (Stratagene), and sequenced using the dideoxy chain termination method of Sanger et al., 1977 and reagents from the Sequenase kit (U. S. Biochemicals).

Cellular Anatomy and Development

Developmental times for embryos and larvae are given in minutes after first cleavage, or hours after hatching, respectively (Sulston and Horvitz, 1977; Sulston et al., 1983). Anatomical and lineal names for cells in *C. elegans* are used as summarized in Wood (1988). Lineal names consist of one or more capital letters denoting an embryonic founder cell (AB, C, D, E, MS, or P4), or a larval blast cell, followed by lower case letters that provide a unique binary code for each descendant. Blastomer ABalp, for example, is the posterior (p) daughter of the left (l) daughter of the anterior (a) daughter of founder cell AB.

Genetic Mosaics

Mosaic embryos were obtained from a strain with genotype *sDp3; dpy-1 pat-3 (rh54) ncl-1*. The small chromosomal fragment, or free duplication, *sDp3* is mitotically unstable. Prospective mosaics could be routinely identified by various phenotypes using a dissecting microscope. Animals with defects in body muscle cells could be selected by abnormalities in body shape or locomotion (e.g., Benthead, Roller). Animals with defects

in gonadal cells could be recognized as L4 larvae. Animals with defects in the excretory cell could be identified as small, pale larvae or adults. Animals with defects in muscle cells used for defecation could be identified as pale, constipated animals, usually as L2 larvae. Animals were mounted for DIC microscopy and examined for defects and the Ncl phenotype to determine where the loss of *sDp3* in the lineage occurred.

Electron Microscopy

Prospective mosaics were first examined by DIC microscopy as L4 larvae to determine their somatic genotypes. Selected individuals were then fixed for electron microscopy as adults using buffered glutaraldehyde followed by osmium tetroxide as described by Sulston et al. (1983). Serial transverse or longitudinal sections were collected through the region of interest. Sections were post-stained with uranyl acetate and lead citrate. Cells were reconstructed from micrographs.

Immunoprecipitations of C. elegans integrin β pat-3 from pat-3(rh96) and pat-3(rh151)ncl-1.

Immunoprecipitations were carried out as described in the previous chapter. Briefly, *pat-3(rh151)ncl-1* animals were individually picked as late L4 larvae or young adults from a population containing *sDp3;pat-3(rh151)ncl-1* and *pat-3(rh151)ncl-1* animals. Animals were identified as *pat-3(rh151)ncl-1* based on the protruding vulva phenotype. N2 animals used as a staged-match control for the *pat-3(rh151)ncl-1* sample were examined by Nomarski microscopy to verify that the young adults did not contain embryos. After collection, worms were washed in M9, frozen in liquid nitrogen, and stored at -80°C until used. Membrane protein extracts were prepared from the samples and biotinylated before immunoprecipitation with the β pat-3 cytoplasmic antibody.

Scoring of onset of paralysis in unc-52;pat-3 double mutants

Double mutant combinations were made between the two viable *pat-3* alleles, *pat-3(rh151)ncl-1* and *pat-3(rh96)*, with four viable alleles of *unc-52* (*e669*, *e998*, *e1012*, and *e1421*) using standard genetic methods. Because *pat-3(rh151)ncl-1(e1865)* is infertile, *dpy-17(y57)* was put *in trans* to mark the non-*pat-3* containing chromosome. To stage worms for scoring the onset of paralysis in *unc-52(e1012)*, *unc-52(e1421)*, *unc-52(e1012);pat-3(rh151)ncl-1* and *unc-52(e1421);pat-3(rh151)ncl-1* animals,

approximately 10 hermaphrodites of the appropriate genotype were placed on petri dishes spotted with bacteria and allowed to lay eggs overnight. The next morning, the hermaphrodites and the hatched larvae were removed and the remaining embryos were allowed to hatch for 3 hours. At this point, which was considered the starting point of the time course, unhatched embryos were removed and the plates were subsequently monitored every few hours for the onset of the Unc phenotype. Worms were scored as Unc (complete paralysis) when they failed to move forward after being touched three times on the tail. All animals were scored by DIC microscopy after being scored as Unc to verify their genotype and exact stage of development.

RESULTS

Mutant isolation, complementation, and mapping

Three independent alleles of the *pat-3* gene, *rh54*, *rh96*, and *rh151*, were isolated following treatment with the mutagen ethylmethane sulfonate (EMS) (Brenner, 1974). *pat-3(rh54)* homozygotes arrest as unhatched larvae with the PAT phenotype (paralyzed arrested elongation at two-fold), *pat-3(rh151)* homozygotes become motile, sterile adults, while *pat-3(rh96)* homozygotes are both motile and fertile. The lethal and sterile alleles were maintained in strains carrying *pat-3(+)* on the free chromosomal duplication *sDp3*.

*Phenotypic rescue of *pat-3* mutants using the *C. elegans* integrin β subunit gene*

The cellular phenotypes of *pat-3* mutants (described below) suggested that the *pat-3(+)* gene product may have a role in cell-matrix interactions in normal development. A comparison of genetic and physical maps of chromosome III revealed that the *C. elegans* integrin β subunit described in the previous chapter was a gene candidate for *pat-3(+)* (Fig. 1). Initially, rescue of the *pat-3(rh54)* embryonic lethal phenotype was obtained by microinjecting a cosmid (ZK1058) containing the entire coding region of the *C. elegans* integrin β subunit gene into the germplasm of *sDp3;dpy-1pat-3(rh54)ncl-1* hermaphrodites. Subsequently, a 9 kb Sal-1 fragment containing the complete coding sequence of the *C. elegans* integrin β subunit gene (see previous chapter) was found to be sufficient to rescue the embryonic lethality of *pat-3(rh54)* as well as post-embryonic phenotypes determined by mosaic analysis (see below). Thus, the integrin β subunit cloned in the previous study has been named *C. elegans* integrin β pat-3.

*Sequence analysis of *pat-3* alleles*

EMS, the chemical mutagen used in these studies, most frequently induces CG-to-TA transitions in double stranded DNA. The hydroxylamine procedure (Cotton et al, 1988), which detects mismatched bases in hybrids of wildtype and mutant DNA, was used to locate the mutations within the *pat-3* gene; subsequently, these regions of DNA were amplified using PCR and the amplified fragments of mutant DNA were sequenced (Fig. 2). Each allele results from a single CG-to-TA transition within the sequence encoding the extracellular domain of *C. elegans* integrin β pat-3. In *rh54*, arginine 190 (CGA) is mutated

Figure 3-1. Position of the *C. elegans* integrin β subunit on linkage group III. The position of the *C. elegans* integrin β subunit was mapped on linkage group III by fingerprint analysis of the 9 kb *sal-1* fragment (Alan Coulson, pers. comm.) By its map position, the cosmid ZK1058 was identified as a candidate for containing the integrin gene; this was verified by Southern blot analysis (see methods). *pat-3* is closely linked and to the right of *dpy-27*.

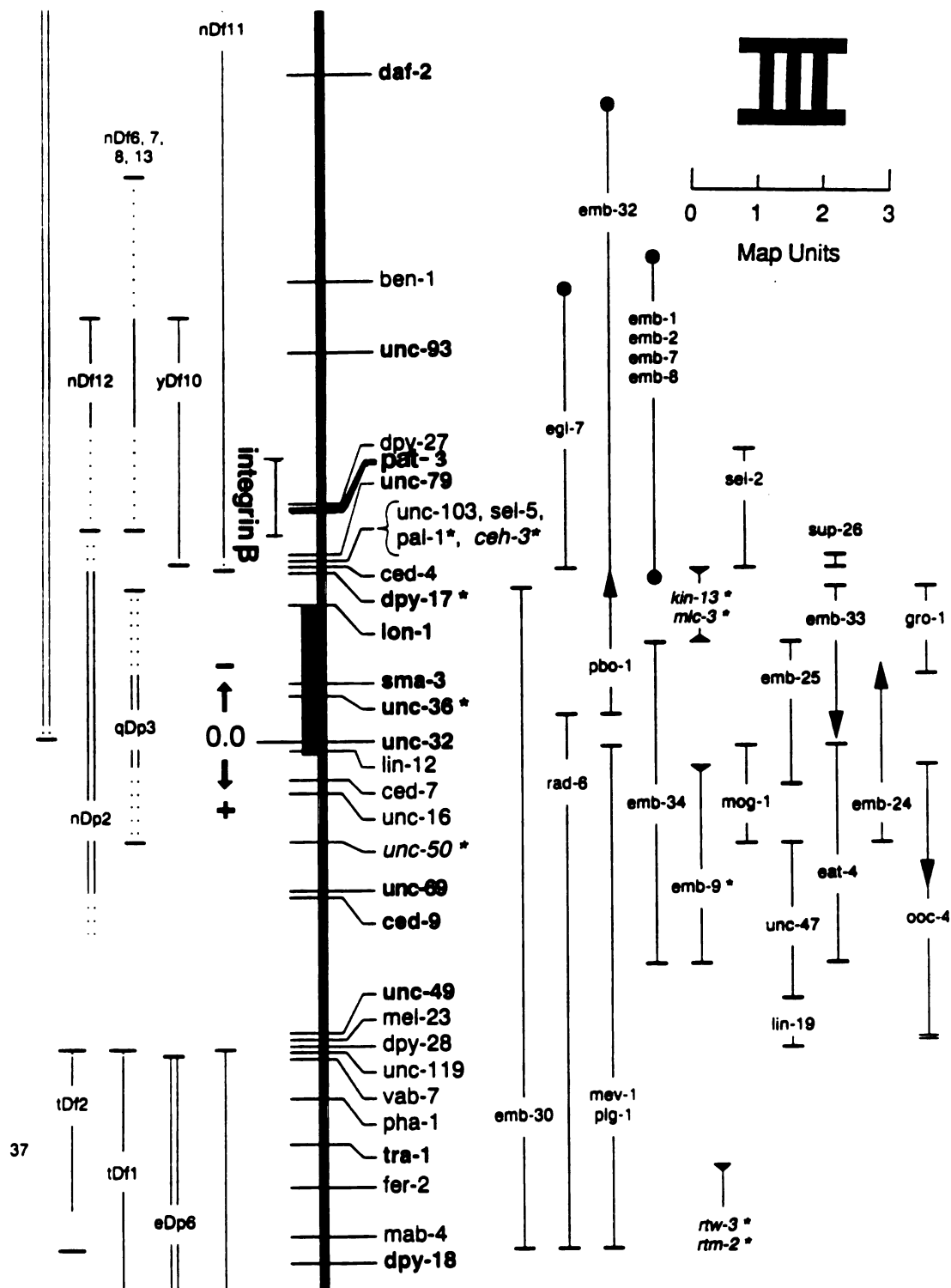
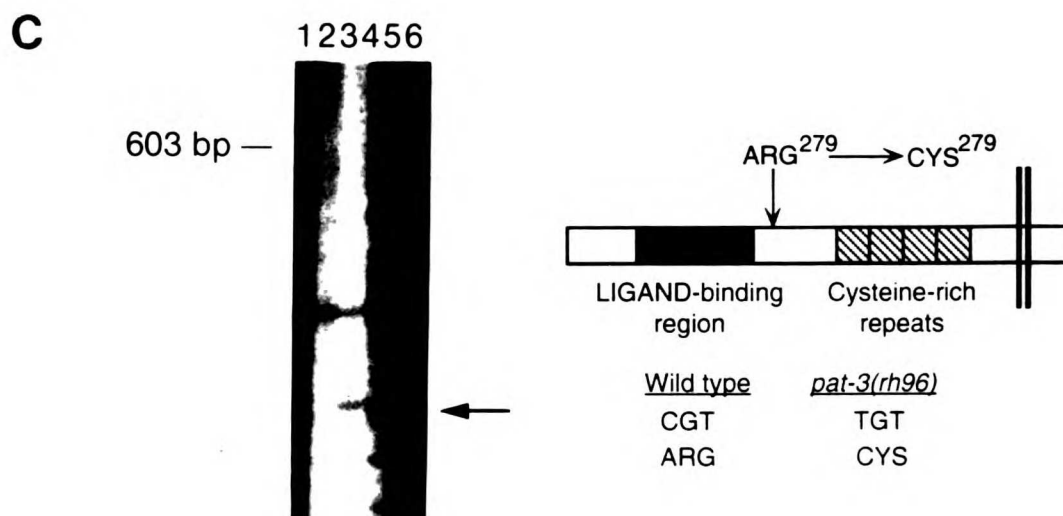
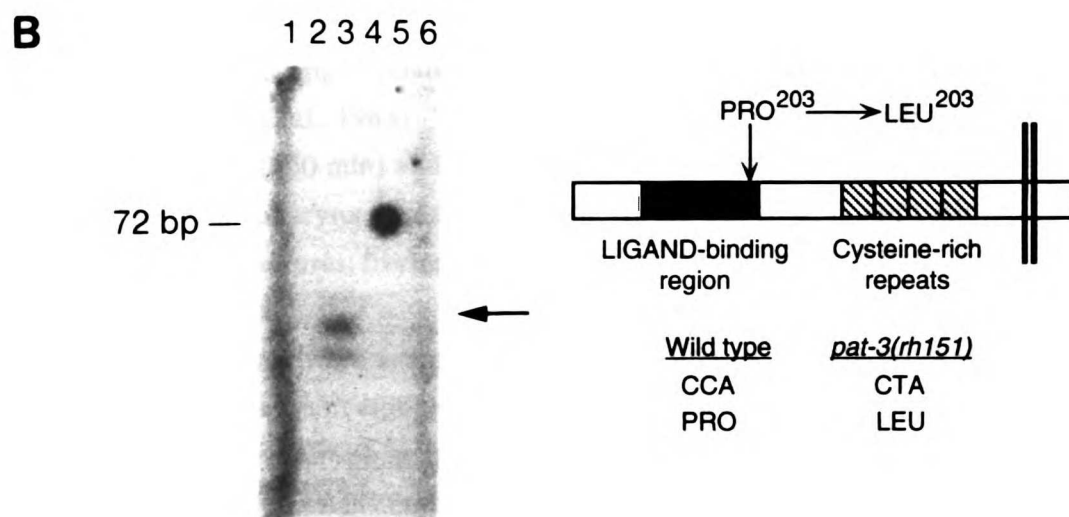
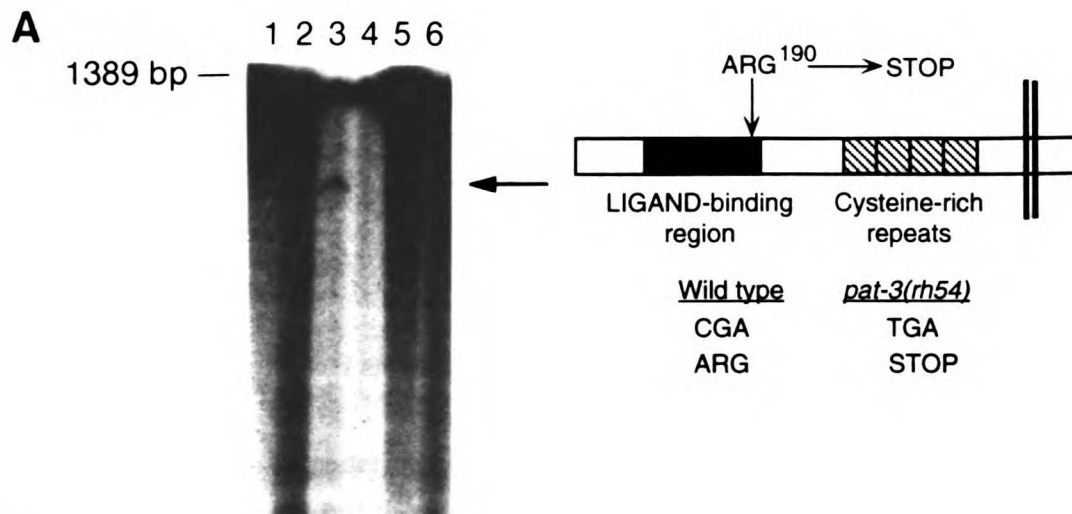


Figure 3-2. Determination of the molecular identity of the *pat-3(rh54)*, *pat-3(rh151)* and *pat-3(rh96)* mutations. The locations of the mutations in the three *pat-3* alleles were narrowed down to a small region of the gene by the hydroxylamine technique (Cotton et al., 1988), which modifies and cleaves double stranded DNA at single base pair mismatches. These regions were subsequently amplified with PCR and sequenced. New fragments were detected autoradiographs of gels in lanes containing ³²P-labeled wild type DNA hybridized with unlabeled mutant DNA (lane 3), but not in lanes containing untreated ³²P-labeled wild type DNA (lane 1), wild type ³²P-labeled DNA hybridized with wild type unlabeled DNA (lane 2), unlabeled wild type DNA hybridized with ³²P-labeled mutant DNA (lane 4), ³²P-labeled mutant DNA hybridized with unlabeled mutant DNA (lane 5) or untreated ³²P-labeled mutant DNA. The mutations in all three alleles were localized to the region between oligos 1 and 2 rev (see Figure 4, previous chapter). (a) *pat-3(rh54)*. (b) *pat-3(rh151)*. (c) *pat-3(rh96)*. The sequence and location of the mutations relative to the integrin β_{pat-3} protein are also shown. Numbers at the left of the autoradiographs indicate molecular weight markers in base pairs.



to the nonsense (amber) codon TGA. As this mutation is located approximately one quarter of the way into the protein, it is likely that no functional integrin is present in *rh54*. Thus, the molecular data is consistent with the genetic data, which classified *rh54* as a null allele based on its severe phenotype. In *rh151*, proline 203 (CCA) is mutated to a leucine (CTA) codon. In *rh96*, the arginine 279 codon (CGT) is mutated to a cysteine (TGT) codon. The location of these mutations in the predicted protein is shown schematically in Fig. 2.

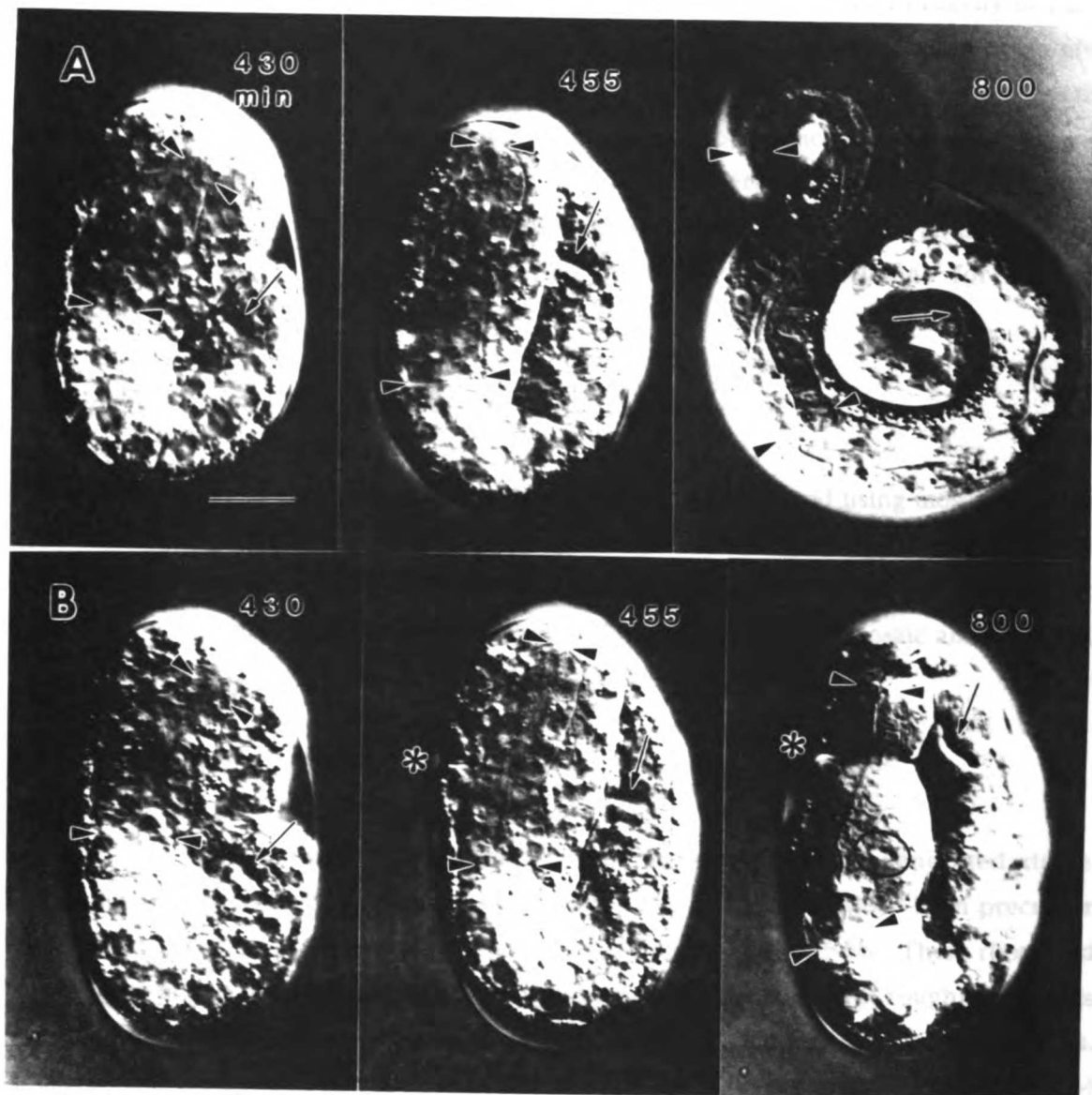
Paralysis and arrest at two-fold elongation of pat-3(rh54)

After closure of the hypodermis (ca. 340 min), wild type embryos begin a period of elongation that continues until the onset of cuticle synthesis (ca. 650 min). During this period, body wall and internal organs increase about four-fold in length and constrict two-fold in diameter. At approximately 430 minutes, the body muscles begin contracting spasmodically (Sulston et al., 1983). These contractions become vigorous and synchronous by two-fold elongation (460 min) and continue throughout embryonic elongation. Shortly before hatching, the pharynx begins spasmodic pumping, and at approximately 800 minutes, the eggshell ruptures, freeing the new larva to move about and feed.

pat-3(rh54) embryos were examined by DIC microscopy to determine the cause of developmental arrest. Mutant embryos develop normally until about 430 minutes (Fig. 3). Embryonic cell lineages, morphogenetic movements (gastrulation, epiboly, neurulation) and the initial elongation of the embryo to the two-fold stage appear completely normal. However, elongation ceases at the two-fold stage (approximately 460 minutes) and the body muscles do not become contractile. In addition, circumferential furrows start to appear after 430 minutes. These furrows, which are permanent, become progressively deeper until cuticle formation. Finally, the sinus at the junction of the excretory cell and its duct becomes swollen with fluid soon after the onset of cuticle formation.

The development of the alimentary tract appears normal until about 460 minutes, when pharyngeal and intestinal, like body wall, elongation cease entirely. About 30 minutes before hatching, the pharynx in mutant embryos begins to pump spasmodically. Although pumping can occur at normal rates (up to 20 cycles per minute), they are relatively ineffectual in distending the intestine. Eggshell softening is delayed, and only about half of the larvae ever hatch. Although the cuticle seems intact, mutant larvae deteriorate rapidly after rupture of their eggshell. In particular, they cannot move about, nor do

Figure 3-3. Comparison of elongation in *pat-3(rh54)* and wild type embryos. DIC microscope images of a wild type embryo (panel A) and a *pat-3(rh54)* embryo (panel B), 100x objective. At 430 minutes, both wild type and *pat-3(rh54)* embryos have reached 1¹/₂-fold in length; *pat-3(rh54)* embryos fail to exhibit small twitching movements that begin in wild type embryos at this stage. By 455 minutes, both embryos have reached two fold in length, but *pat-3(rh54)* embryos fail to exhibit the vigorous movements observed in wild type embryos. At 800 minutes, wild type embryos have completed elongation and are hatching, whereas *pat-3(rh54)* embryos have failed to elongate beyond the two fold length. However, structures such as the pharinx (indicated by arrowheads in all photographs) and the anus (arrow) have formed.



they feed. The direct cause of developmental arrest was not apparent.

Maternal effects

We examined progeny of germline mosaics to learn whether maternal expression (*mat*) of *pat-3(+)* during oogenesis contributes to normal embryogenesis. Self-progeny of *P₃(-)* or *P₄(-)* mosaics, genetically *mat(-) zyg(-)*, were indistinguishable from *mat(+)* *zyg(-)* progeny of *P₄(+)* hermaphrodites. In particular, these embryos complete morphogenetic movements and initial elongation normally, followed by appearance of the Pat phenotype. Viable cross-progeny were obtained from mating *P₄(-)* hermaphrodites with wild type males. These *mat(-) zyg(+)* larvae were indistinguishable from wild type. Thus maternal gene expression, if it occurs, is neither necessary nor sufficient for normal embryogenesis.

Post-embryonic phenotypes of pat-3(rh54)

The post-embryonic phenotypes of *pat-3(rh54)* were determined using mosaic analysis (see methods, also Fig. 4 and 7). The defects for each tissue or cell examined are described in the following section. A short description of wild type anatomy is also presented as background for the description of the defects found in mosaic animals. The results of the mosaic analysis are summarized in Table 1.

Body wall muscles

In wild type animals, a total of 81 body muscle cells are generated during embryogenesis from founder cells AB, MS, C and D (Sulston et al., 1983). Each precursor generates 1, 28, 32, and 20 mononucleate body muscle cells, respectively. These rhomboid cells are arranged in four longitudinal bands along the body wall, with roughly two cells abreast in each band. The cell positions are essentially invariant between individuals. Muscle cells from different precursors interleave considerably, but MS (excepting M below) and D descendants are positioned in the head and trunk, while C descendants are positioned in the trunk and tail. At first molt, 14 additional body muscle cells from mesoblast M (MSapaapp) join the muscle bands in the tail. Cellular processes called muscle arms project to nearby motoaxons in the nerve ring and nerve cords where they form neuromuscular junctions. Body muscles also form gap junctions with other muscle and non-muscle (e. g., GLR, hmc) cells.

Each muscle quadrant is enclosed in a basal lamina and supported along its entire length by hypodermal cells forming the body wall (White et al., 1976). Body muscles are polarized by contact with the hypodermis (Waterston et al., 1980): Myofilaments assemble only along the surface apposed to the hypodermis, while the cytoplasmic region distal to the hypodermis contains organelles (e.g., nucleus, mitochondria, ribosomes, ER) excluded from the myofibrillar lattice. The myofilaments are aligned longitudinally but offset from their neighbors, giving each muscle band an oblique striation (Rosenbluth, 1965). Thin filaments are anchored to terminal plaques (half-I-bands) at cell borders or to periodic Z-disc analogs, the dense bodies (I-bands), within the sarcomere. Both are junctional sites, linking thin filaments to the sarcolemma and the extracellular matrix. Thick filaments are anchored to the sarcolemma by a continuous junctional complex, the M-line.

In *pat-3(rh54)* mosaic animals, losses of *sDp3* in AB, MS, C, and D produce viable animals that develop to adulthood. AB(-) larvae have normal body shape, but the others have characteristic deformities resulting from a regional failure of embryonic elongation (Fig. 4). In MS(-) mosaics, the head is short and bent ventrally, while the trunk and tail are normal. In D(-) mosaics, the head and trunk are short and thick and the trunk is variably scalloped or furrowed, while the tail is normal. In C(-) mosaics, the head and trunk are normal, while the tail is short and thick. In unilateral mosaics, e.g., MSp(-), Cp(-), or Dp(-), elongation is almost complete, but the body usually has a permanent twist in the affected region. Twists vary both in handedness and magnitude (up to 180°) even among mosaics of identical somatic genotype.

Analysis of mosaic animals suggests that β pat-3 expression in body muscles is required for normal embryonic elongation. This is most obvious for founder cell D, which generates exclusively body muscle cells. To test this idea, we screened directly for larvae with deformities of body shape under a low-magnification microscope. Without exception, a body muscle precursor was affected in each of the deformed larvae examined. In particular, larvae with just 5-10 mutant muscle cells, e.g., MSppp(-), Cppp(-), or Dp(-) mosaics, can have mild but observable deformities. MS(-), C(-) and D(-) mosaics can form viable larvae, showing that embryos can tolerate as many as 20-32 mutant muscle cells. Viable P₁(-) and P₂(-) larvae were never observed; presumably these mosaics either die or arrest growth at hatching. Thus, embryos cannot tolerate simultaneous loss of β pat-3 expression in both C and D descendants, which comprise 52 of the 81 embryonic body

TABLE 1. SUMMARY OF DEVELOPMENTAL FUNCTIONS OF INTEGRIN β pat-3 FROM PAT-3(rh54) MOSAIC ANALYSIS.

ORGAN / TISSUE / CELL	ORIGIN	β pat-3 FUNCTIONS	NOT REQUIRED
MUSCLES			
• anal depressor	ABppppppap	Cell attachment.	
• body wall	AB, MS, C, D	Cell attachment, organization of sarcomere.	
• intestinal	ABppppppaa, MSppaap		
• sphincter	ABppppppap		
• sex-specific muscles	MSapaap = M	Cell attachment, migration of sex mesoblast (SM).	
GONAD			
• embryonic mesoblasts (Z1, Z4)	MSppaap, MSapaap		Cell migration, rudiment assembly and body wall attachment.
• distal tip cells		Epithelialization.	Cell migration, germline proliferation.
• ovarian sheath cells		Migration, epithelialization.	
• anchor cell		None.	
PHARYNX			
• glands: g1-g2	MSaa, MSpa	None.	
• marginal cells: mc1-mc3	ABa, MSaa, MSpa	None.	
• muscles: m1-m8	ABa, MSaa, MSpa	None.	
• neurons	ABa, MSaa, MSpa	None.	
• valve: vp1-vp3	MSaa, MSpa	None.	
MISC. MESODERMAL CELLS			
• coelomocytes/cm		None.	Cell migration
• head mesodermal cells		None.	Cell migration, process outgrowth.
• mesogleia (GLR)		?	
HYPODERMIS			
• hyp1-hyp11	AB, C		
• H0-H2, V1-V6, T	AB		
• P1-P12	ABp		
• rectal: B,Y,F,U,K,rep,vir	ABp		
NERVOUS SYSTEM			
• neurons	AB, C		
• ALM neuroblasts	ABappaap, ABppppappa	None.	
• CAN neuroblasts	ABapaapa, ABalappappa	Cell migration.	
• HSN neuroblasts	ABlappppappa, ABppppppappa	Cell migration.	
• Q neuroblasts		Final cell position.	
• PLM		Cell migration.	
• sensilla: sheath, socket	AB	Axon guidance/outgrowth.	
EXCRETORY SYSTEM		None.	
• excretory cell	ABlppppaap		
• duct cell	ABpapaapa	Canal outgrowth.	
• pore cell	ABppapaapa (G1), ABpapaapa (G2)	None.	
• gland cells	ABlppapapa, ABppppapapa	None.	
INTESTINE	E	None.	
GERMLINE	P4	None.	

Figure 3-4. Nomarski montage of *pat-3(rh54)* mosaic animals with loss of *sDp3* in the MS, D and C founder cells. Animals with losses in the MS, D, and C lineages could easily be detected by bent heads, shortened trunks, or shortened tails, respectively.

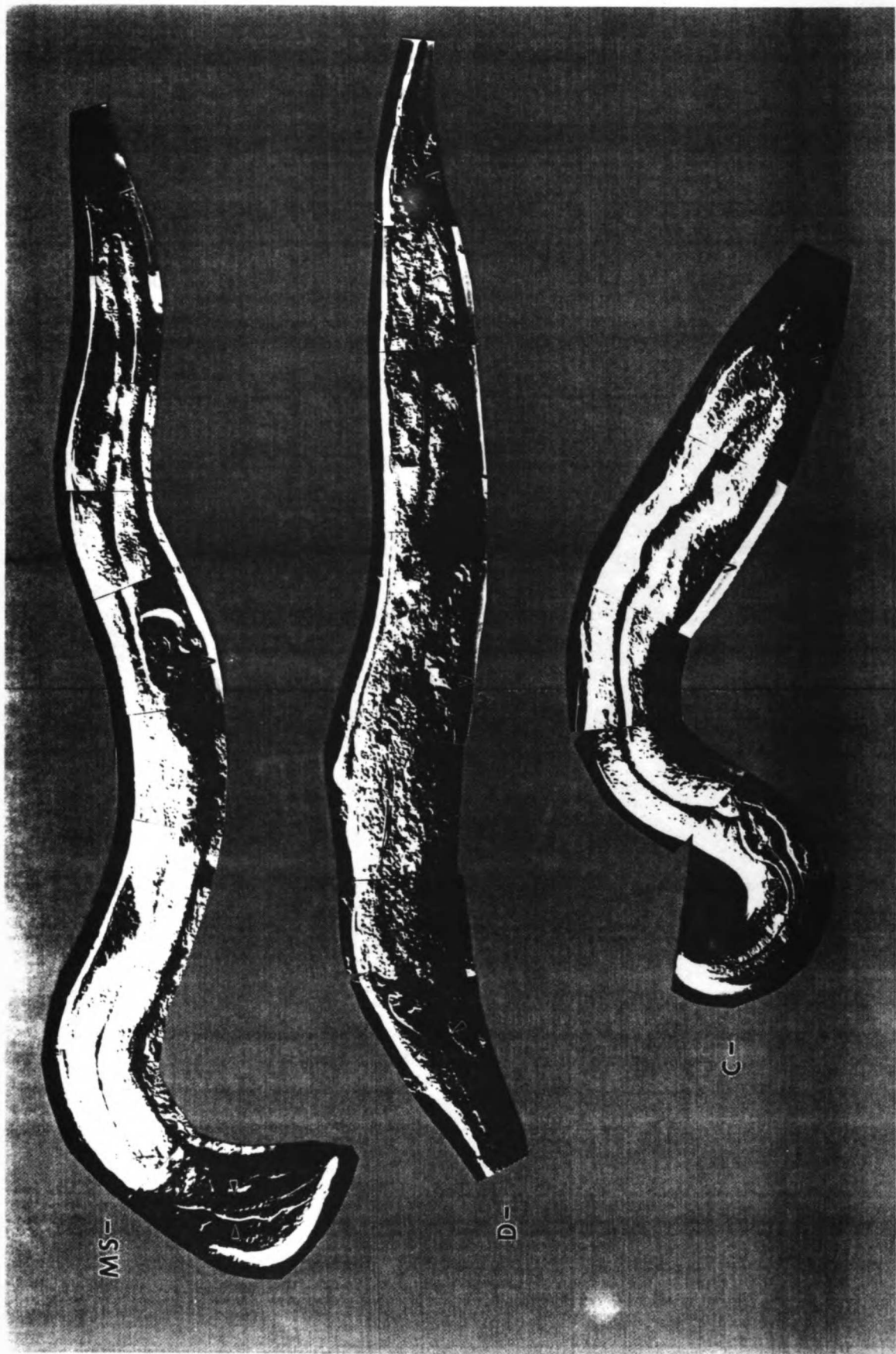
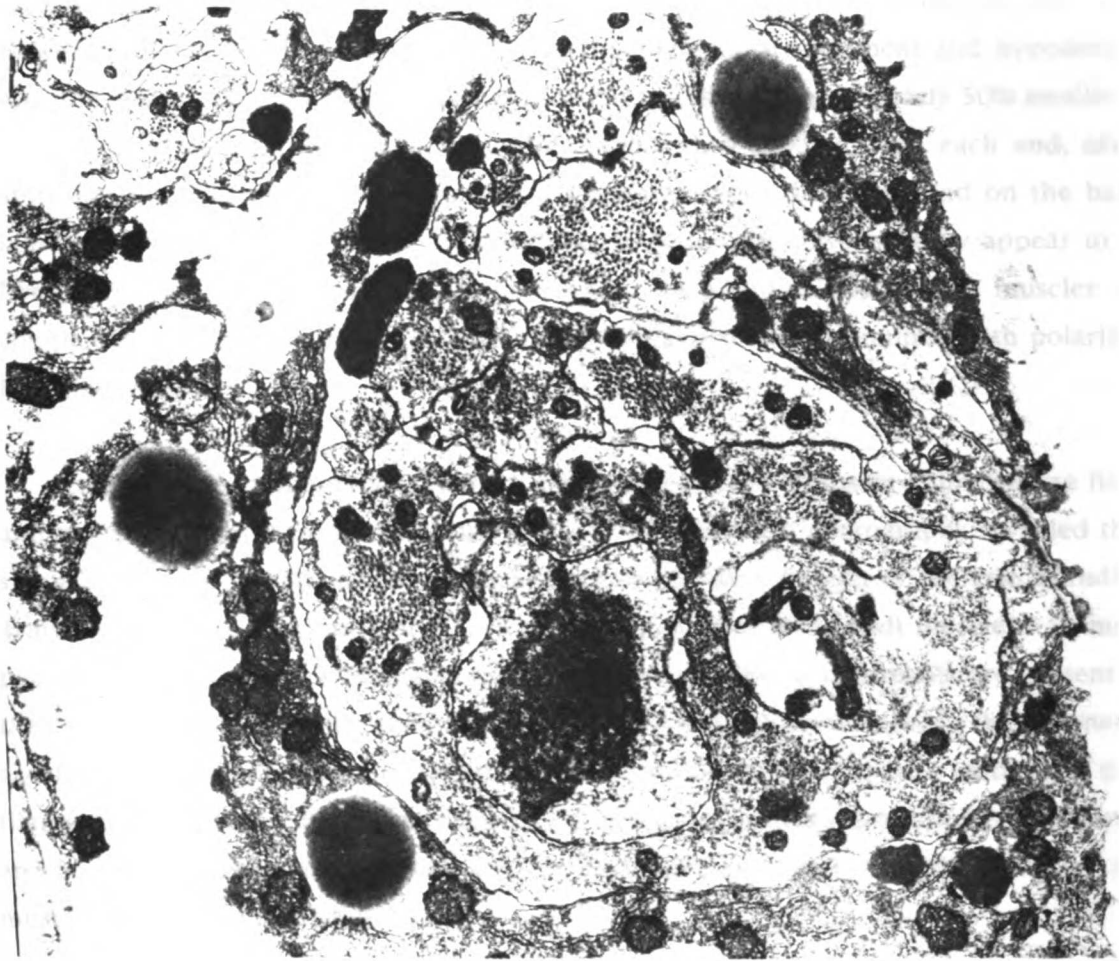


Figure 3-5. Electron micrograph of *pat-3*(-) body wall muscle. In contrast to wild type body wall muscles, *pat-3* mutant muscles are not apposed to the cuticle (right side of photograph) and spaces exist in between muscle cells. The cells are also smaller than wild type. Microfilaments can be seen, but higher magnification shows that they are disorganized (not shown). A Ncl(-) nucleus, containing a large nucleolus (electron dense material) can be seen. The basal lamina to the left of the cells is present in large amounts and is disorganized, in contrast to the thin sheet surrounding the cells seen in wild type. Wild type muscle can be seen in Figure 6 for comparison (lower left hand corner).

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wall muscles.

Body wall muscle cells were examined in living adult mosaics by DIC and polarized light microscopy. Individual *pat-3(-)* muscle cells are easily distinguished from neighboring *pat-3(+)* cells by their enlarged nucleoli (Ncl phenotype). Muscle cell lineages are evidently unaltered in AB(-), MS(-), C(-) and D(-) mosaics. Moreover, *pat-3(-)* body muscle cells are fully viable and assume roughly wild type positions along the body wall. However, the overall shape of each cell, its myofilament arrangement and hypodermal attachments are dramatically reorganized. Mutant cells are approximately 50% smaller in volume than wild type. Roughly spindle-shaped, they taper toward each end, often splitting into several smaller processes. The mutant cells do not spread on the basal lamina, nor do they compress the underlying hypodermis; however, they appear to be firmly tethered to the body wall at their ends. In addition, the mutant muscles are completely non-contractile and exhibit no birefringence when examined with polarized light microscopy.

After an initial examination by light microscopy, selected mosaic animals were fixed and examined by electron microscopy (Fig. 5). The electron micrographs revealed that small bundles of myofilaments, which are present in each branch of the cell including arms to the nerve ring and cords, are present in mutant body wall muscles. In most places, the thin and thick filaments are interdigitated. The myofilaments are present in the central cytoplasm rather than adjacent the sarcolemma; however, only thin filaments are found at sites of bundle splitting or the terminal 1-2 microns. Although thin filaments end adjacent to the sarcolemma, no vestiges of terminal plaques were observed, and no dense bodies were observed. Finally, the basal lamina that normally surrounds the muscle quadrant is disorganized.

Anal depressor and intestinal muscles

Four specialized muscle cells, the intestinal muscles (ABplpppppaa, MSppaapp), the sphincter muscle (AB prpppppap), and the anal depressor muscle (ABplpppppap), together with the body wall muscles, are required for defecation. Losses of *sDp3* in AB result in small pale adults with a distended intestine. The intestinal abnormality, as well as most of the small size and palor of the animals, focus the loss of *sDp3* to ABplppppp, which generates the anal depressor muscle and sphincter muscle. In these animals, the morphology and the ventral attachments of anal depressor muscle are disrupted.

Information about the defects in morphology and attachments of mutant intestinal and sphincter muscles is not currently available.

Sex-specific muscles

During the second larval stage, the two sex myoblasts (SM) migrate to a position near the vulva and divide to produce 16 sex-specific muscles: eight uterine muscles, used to squeeze embryos into and out of the uterus and eight vulval muscles, used to open the vulva during egg-laying. Losses of *sDp3* in the progenitor of SM, M (MSapaapp), result in defects of the migration of SM, and a failure of its descendents, the vulval muscles, to attach. Information about defects in the uterine muscles is not available.

Pharynx

The pharynx, and its associated nervous system, is a simple myoepithelial organ that forms the initial segment of the alimentary tract (Albertson and Thomson, 1976). The myoepithelium is comprised of two classes of structural elements, muscle cells and marginal cells, and two types of gland cells. A typical cross-section through the pharynx shows 3 muscle cells interspersed with 3 marginal cells. These cells secrete a cuticle from their apical (luminal) surfaces and a thick basal lamina bounding the pharynx. In the muscle cells, radial myofibrils attach to the cuticle and basal lamina at each end. These myofibrils are not striated but represent a single sarcomere with no overt M-line specialization. In the marginal cells, radial tonofilaments attach to the cuticle and the basal lamina at each end. Both tonofilaments and myofibrils attach to the basal lamina at specialized sites resembling vertebrate hemidesmosomes. The apical membrane of marginal cells has similar attachment sites between tonofilaments and cuticle, while the entire apical membrane of muscle cells is darkly specialized.

β pat-3 expression is not required for pharyngeal development or function. The cytoarchitecture of pharyngeal glands, muscles, and marginal cells is normal in AB(-) and MS(-) mosaics. Moreover, these individuals feed, grow and molt at nearly wild type rates. Although the pharynx is abnormally short and bent ventrally in MS(-) mosaics (Fig. 4), the pharyngeal deformity in MS(-) mosaics most likely results from failed body elongation because its morphology is normal in MSaa(-) and MSpa(-) mosaics. Furthermore, although the pharynx is also short in D(-) mosaics (Fig. 4), no pharyngeal cells derive from D. Thus, in these animals, the failure of pharyngeal elongation is clearly secondary to the

defect of body elongation.

Somatic gonad

Complete information regarding the function of β pat-3 in the formation of the somatic gonad is not currently available as a thorough understanding of the morphological defects in mosaic animals will require analysis by electron microscopy. However, DIC microscopy revealed that β pat-3 is required for the migration and epithelialization of the ovarian sheath cells, the epithelialization of the distal tip cells, and the epithelialization of cells that form the uterus. β pat-3 expression is not required for the migration, rudiment assembly, and body wall attachment of the embryonic mesoblasts, Z1 and Z4, the migration and germline proliferative functions of the distal tip cells, or the functions of the anchor cell.

Germline

Germline mosaic animals with losses of *sDp3* in the progenitor cell P_3 , which generates the muscle founder cell D and the germline founder cell P_4 , were examined to learn whether β pat-3 expression is required for any aspect of gametogenesis or fertilization. Prospective $P_3(-)$ mosaics were first identified by their abnormal body shape (due to loss of *sDp3* in D) and then examined by DIC microscopy to confirm that D-derived body muscles were phenotypically Ncl Pat. Of five such hermaphrodites, three hermaphrodites produced both non-Pat and Pat embryos, indicating a $D(-) P_4(+)$ somatic genotype. The other two hermaphrodites, which produced exclusively Pat embryos, were evidently $P_3(-)$ mosaics. Prospective $P_4(-)$ mosaics were identified by examining progeny, either while inside gravid hermaphrodites, or after deposition on the culture dish. Of nearly 1000 randomly selected, self-fertile hermaphrodites, four individuals failed to produce any non-Pat progeny and were evidently $P_4(-)$ mosaics. Both classes of germline mosaics, i.e., $P_3(-)$ and $P_4(-)$, are highly self-fertile. Moreover, germline proliferation, meiosis, spermiogenesis, oogenesis, and fertilization appeared completely normal by DIC microscopy. Thus, β pat-3 expression in germline cells is not required for normal germline development.

The excretory system

The excretory cell along with its accessory gland, duct and pore cells, form a primitive

renal epithelium called the excretory system (Nelson et al., 1983). These cells, which are evidently ectodermal in origin, all descend from blastomer ABp (Sulston et al., 1983). After epiboly, the excretory cell rests on the ventral hypodermis in the head. During embryogenesis, a cellular process called a canal invades each of the two lateral ridges. Branching once, the canals migrate anteriorly and posteriorly between the hypodermis and a basal lamina, extending nearly the entire length of the animal. These migrations are not completed until several hours after hatching (Matthew Buechner, pers. comm.). A continuous matrix, secreted from the excretory cell itself, covers the canals where they bridge between ventral and lateral hypodermal ridges. The mature excretory cell has an H-shaped geometry, i.e., four longitudinal canals cross-bridged by the cell body. The apical and basal surfaces of the excretory canals have characteristic specializations. Membrane-bound organelles called canaliculi are present on the apical surfaces, and gap junctions with the hypodermis are found along the basal surface.

In *pat-3(rh54)* embryos, processes from the excretory cell invade the lateral ridges but fail to migrate any further along the hypodermis. The defect of process outgrowth is autonomous to the excretory cell (ABp|pappaap) and completely penetrant. In about half of these mosaics, only one lateral ridge is invaded. The excretory cell process and hypodermal cells mutually invaginate, increasing the area of cell-cell contact. The overall shape of the excretory cell and its internal channels are dramatically reorganized in *pat-3* mosaics (Fig. 6). While the total cell volume appears unchanged, the cell body is greatly enlarged at the expense of the processes. Based on serial reconstruction, this system has a branched, tree-like topology, and forms an open connection with the excretory duct. A system of convoluted channels and their associated canaliculi occupies most of the central cytoplasm. Other organelles, including the nucleus, mitochondria, ribosomes, the endoplasmic reticulum, and Golgi apparatus, are largely excluded from the channel region, concentrating instead in the peripheral (basal) cytoplasm. An apparent exception, multivesicular bodies are found throughout the cytoplasm.

In mosaic animals, the excretory cell forms normal gap junctions with the hypodermis. Unexpectedly, there are also numerous deep infoldings of the cell membrane where the excretory cell forms large gap junctions with itself (Fig. 6). Where infolded membranes separate from another, a well-defined basal lamina is often evident. Occasional gap junctions between excretory cell and neurons have also been observed in these mosaics; these are not observed in wild type.

Figure 3-6. Electron micrograph of a *pat-3(-)* excretory cell. The excretory cell, which normally sends out processes along both sides of the animal, fails to do so when *pat-3(-)*. Instead, the extra membrane is invaginated into the cell, resulting in a large, swollen cell. Arrows indicate the apical, or luminal surfaces of the processes. The small vesicular-like and tube-like openings are the canaliculi, which are normally present in the processes. The cell labeled "pore cell" should read duct cell.



The excretory gland, duct, and pore cells were also examined in relevant mosaics (e.g., ABp(-)) using DIC and electron microscopy. Excepting the defects described above, which we attribute to failed outgrowth of the excretory cell processes, *βpat-3* has no other apparent function in the assembly of the excretory system.

The nervous system

The somatic nervous system is comprised of neurons and accessory cells supported on the hypodermis. *βpat-3* is apparently not required in the nervous system for normal neuronal development and behaviors including locomotion, defecation, pharyngeal pumping, touch avoidance, and egg-laying. However, *βpat-3* is required for the embryonic migrations of the bilateral homologs CANR and CANL (canal associated neuron, function unknown), ALMR and ALML (touch neurons), and the final positioning of HSNR and HSNL (responsible for egg-laying behavior). In addition, *βpat-3* is required for the post-embryonic migrations of the descendants of the Q neuroblast, AQR, AVM, SDQR and PQR. The strategy used to obtain mosaic animals with losses of *sDp3* in these neurons is outlined in Fig. 7.

In wild type, CANR, CANL, ALMR and ALML are born in the anterior of animals and migrate to lateral positions in the mid-body region during embryogenesis. In *pat-3(rh54)* mosaics, the final position of these neurons is not wild type (Fig. 8; complete data not available). The HSN neuron, which is responsible for egg-laying behavior, is born in the posterior of the animal and migrates to a position near the gonad primordium during embryogenesis. In *pat-3(rh54)* mosaics (loss in ABplappppappa and Apprapppappa, thus autonomous to HSN), HSN reaches the gonad primordium, but its final position is often further anterior or more dorsal than its wild type position (Fig. 8).

The Q neuroblast and its descendants migrate during L1, with cell divisions and programmed cell deaths occurring during the migration. The Q progenitor cell is born on both sides of the animal near its sister cell, the lateral hypodermal seam cell V5. On the right side, the final positions of the three Q descendants are in the anterior of the animal: AQR near the pharynx, and AVM and SDQR located ventrally and dorsally, respectively, and posteriorly of the lateral seam cell V1 (Fig. 9). On the left side, PVM and SDQL remain approximately where they are born, near V5, but PQR migrates posteriorly to the tail (Fig. 9A and B). In mosaic animals with losses of *sDp3* in ABp through ABprapapaaa,

Figure 3-7. Strategy used to isolate mosaic animals with *pat-3*(-) neurons. AB, which is one of the two founder cells generated in the first embryonic division, produces mostly hypodermal and neural cells, and a few muscle cells. A simplified version of the AB lineage is presented with the relevant descendents listed at the bottom of the figure. The AB lineage generates all of the neurons that migrate embryonically and post-embryonically: ALM, and CAN are produced in the ABa lineage, and HSN and Q are produced in the ABp lineage. Loss of *sDp3* in the anal depressor muscle (ABp1ppppppap) or any of its parental cells results in animals that are pale and constipated, a phenotype that can be detected using a dissecting microscope. These animals were mounted for Nomarski microscopy and scored for *pat-3* phenotypes including the detachment of the anal depressor muscle, failure of outgrowth of the excretory cell processes, the positions of the Q descendents, CAN, ALM, and HSN, and the trajectory of the PLM axon. The nuclei of these cells were also scored for the Ncl phenotype to verify the loss of *sDp3*. In addition, other nuclei, such as the amphid neurons ASK, ADL and ASI, and the hypodermal nuclei hyp8/9 and hyp10, were scored to determine more precisely where the loss of *sDp3* had occurred.

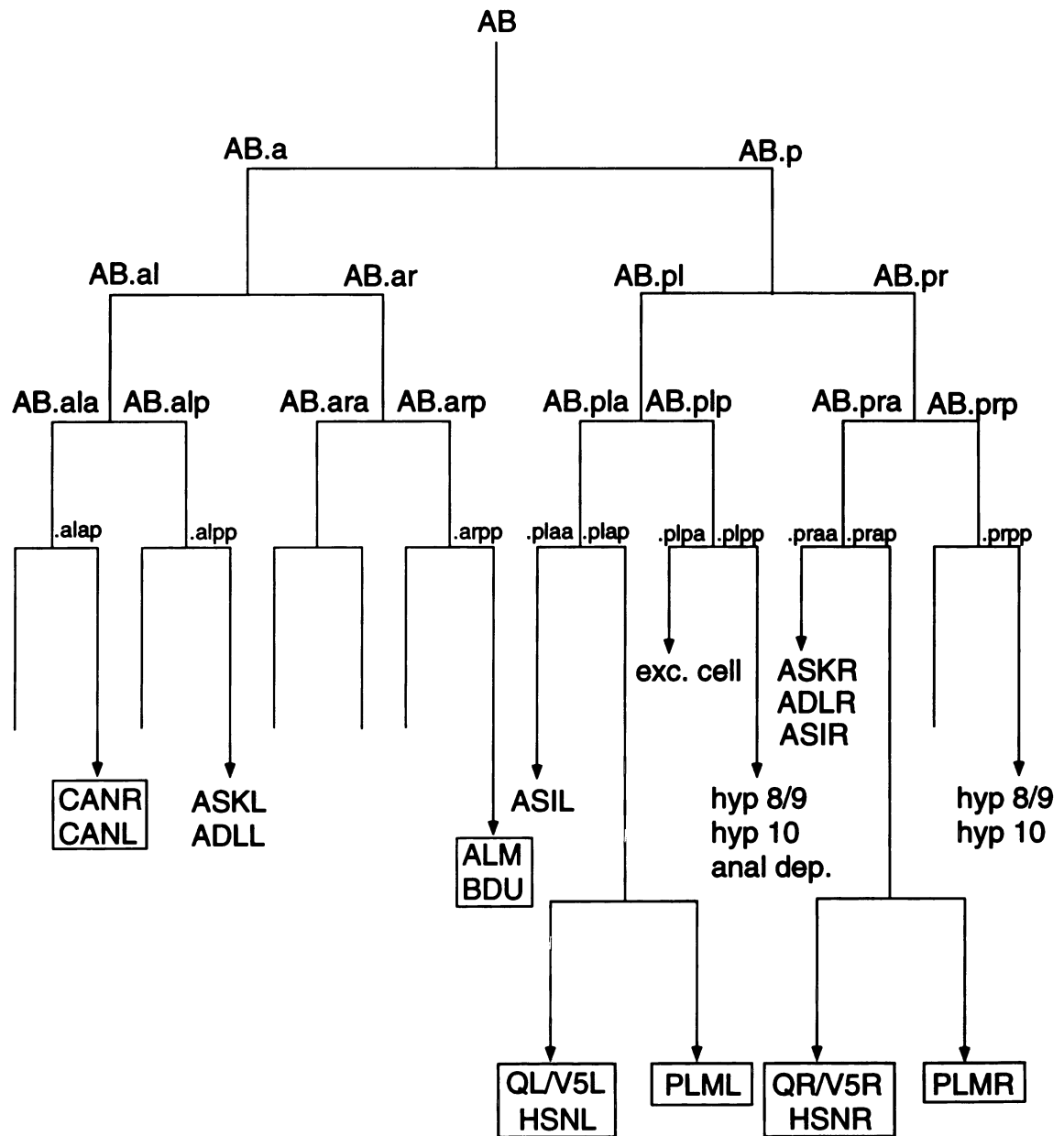
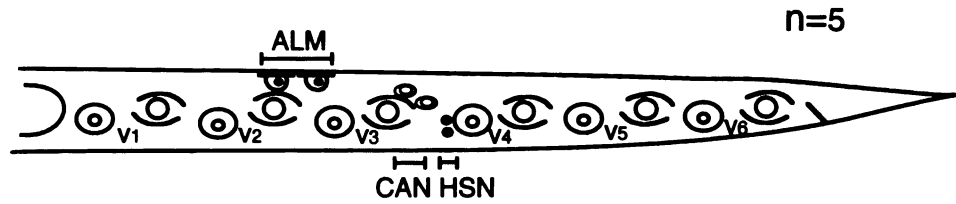
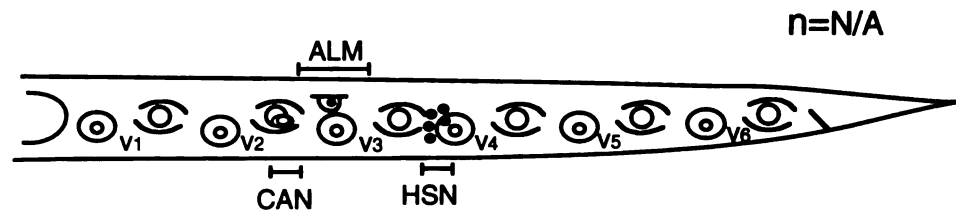


Figure 3-8. Embryonic neuroblast migrations in *pat-3(rh54)*, *pat-3(rh151)*, and *pat-3(rh96)*. The final positions of CAN, ALM and HSN in all three *pat-3* alleles and wild type animals were determined using the lateral hypodermal nuclei, V1-V5, as landmarks. These nuclei are represented by concentric circles, shown in the L1 pattern. Animals were scored after the division of the V1-6 and after the P nuclei descended into the nerve cord. The positions of these neurons were determined in L1 animals for wild type, *pat-3(rh151)* and *pat-3(rh96)* animals, and in L2 or later for *pat-3(rh54)* mosaic animals. The positions of CAN, ALM and HSN are approximately equivalent on the both sides of the animal, therefore data from both sides are shown together. The anterior of the animal is to the left.

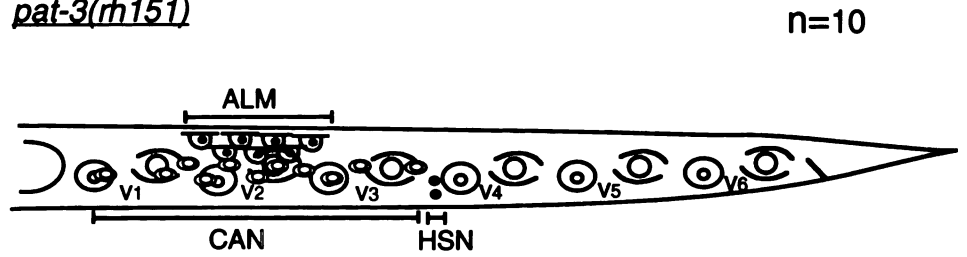
wild type



pat-3(rh54) mosaics



pat-3(rh151)



pat-3(rh96)

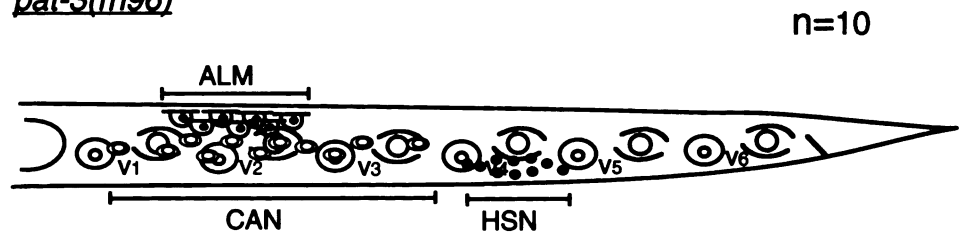
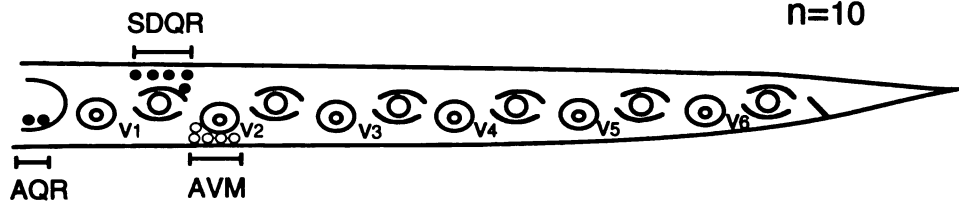


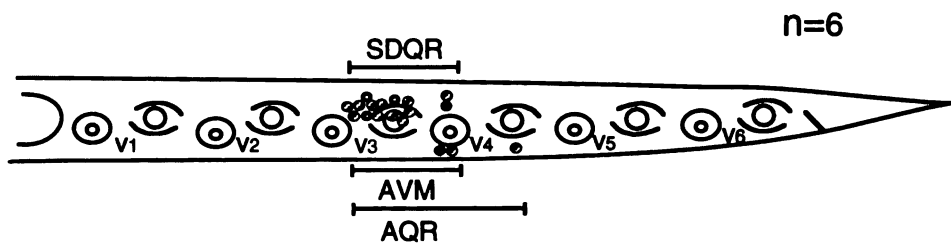
Figure 3-9. Post-embryonic neuroblast migrations in *pat-3(rh54)*, *pat-3(rh151)*, and *pat-3(rh96)*. The final positions of the Q descendants in all three *pat-3* alleles were determined using the lateral hypodermal nuclei, V1-V5, as landmarks. These nuclei are represented by concentric circles, shown in the L1 pattern. Animals were scored after the division of the V1-6 and after the P nuclei descended into the nerve cord. The positions of these neurons were determined in L1 larvae for *pat-3(rh151)* and *pat-3(rh96)*, and in L2 or later for *pat-3(rh54)* mosaic animals. The anterior of the animal is to the left. (a) The final positions of the Q descendants AQR, SDQR, and AVM on the right side of the animal. AQR, grey dots, SDQR, black dots, and AVM, unfilled dots. In *pat-3(rh54)* mosaics, these neurons were grouped together and thus could not be individually identified. Therefore, all dots are filled with stripes. (b) The final positions of the Q descendants PQR, SDQL, and PVM on the left side of the animal. PQR, grey dots, SDQL, black dots, PVM, white dots. In actuality, SDQL could not be distinguished from PVM.

A RIGHT SIDE

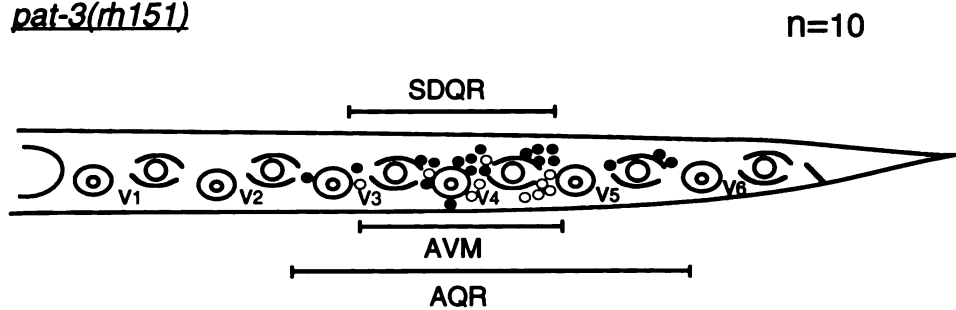
wild type



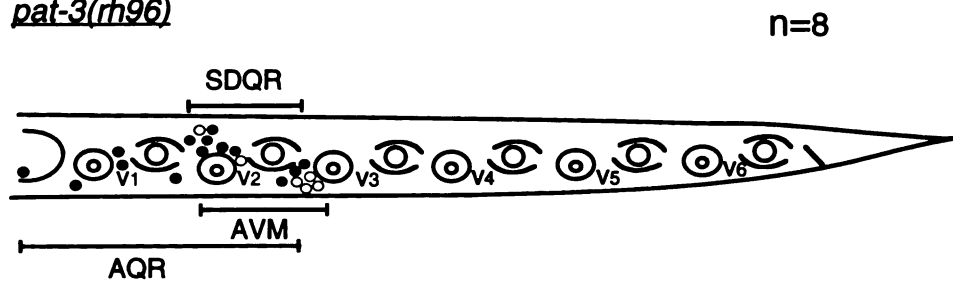
pat-3(rh54) mosaics



pat-3(rh151)



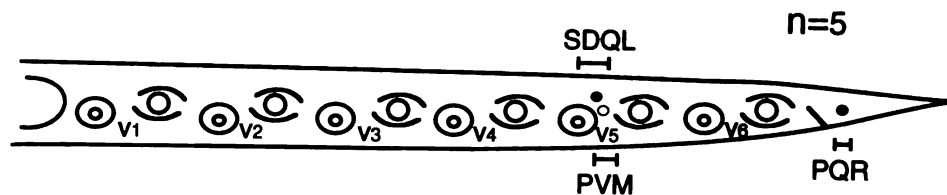
pat-3(rh96)



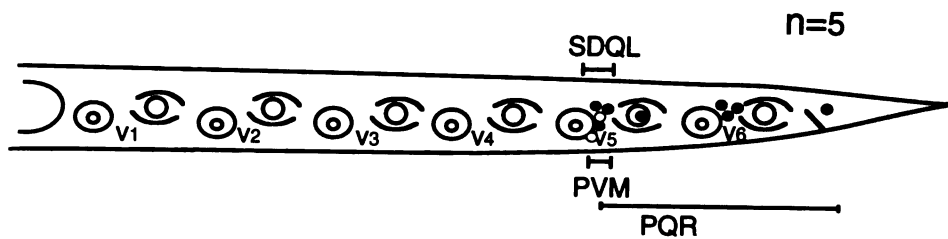
B

LEFT SIDE

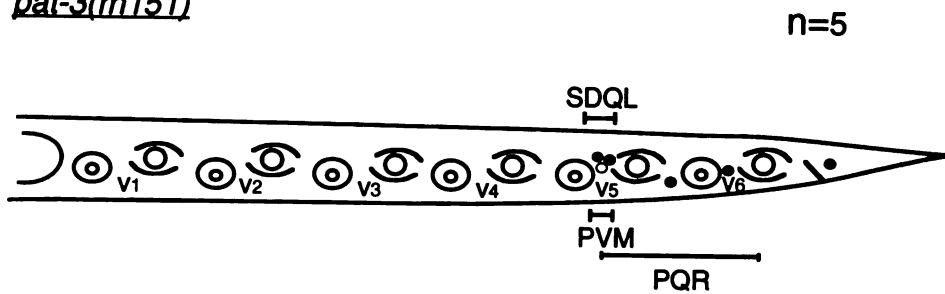
wild type



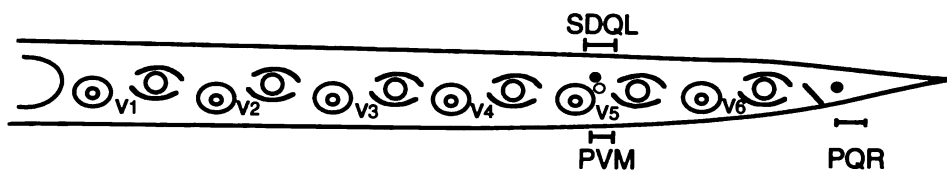
pat-3(rh54) mosaics



pat-3(rh151)



pat-3(rh96)



and thus autonomous to QR (ABprapapaaa), the final positions of SDQR, AVM, and AQR are clustered in between the lateral hypodermal cells V3 and V4 (fig. 9A). On the left side, PQR did not always complete its posterior migration (Fig. 9B).

Finally, the PLM axon, which in wild type animals extends laterally from the tail until it reaches the vulva where it turns ventrally, was observed in mosaic animals with loss of *sDp3* in ABp to turn ventrally more posteriorly than in wild type (data not shown). The position at which it turned ventrally was variable, ranging from just anterior to the anus to approximately halfway to its wild type turning point. The autonomy of this defect has not been determined.

Intestine

The intestine is a simple cylindrical epithelium that forms the posterior segment of the alimentary tract (Sulston and Horvitz, 1977; Sulston et al., 1983). It connects at opposite ends with the pharynx and rectal hypodermis. Specialized for adsorption, its luminal surface is lined with microvilli. Blastomer EMS is the mother of intestinal founder E and mesodermal founder MS. In larvae, the nuclei of intestinal cells become large and highly polyploid (Hedgecock and White, 1985).

The development of the intestine appears essentially normal in *pat-3* embryos. Intestinal cells form a regular cylinder connected to pharyngeal and rectal epithelia. The organ has a well-formed lumen and bounding basal lamina. Since Ncl and non-Ncl cells are not readily distinguished in the intestine, we could not identify E(-) mosaics directly. Instead, prospective EMS(-) mosaics were first identified by abnormal shapes of their body and gonad and subsequently examined by DIC microscopy to confirm that MS-derived cells were phenotypically Ncl Pat. Assuming that intestinal mosaics are viable, we expect roughly equal numbers of E(+) MS(-) and E(-) MS(-) larvae. The intestine appeared completely normal in all mosaics sampled. Thus, β *pat-3* expression is probably not required for intestinal development.

Hypodermis

The hypodermis is a simple epithelium that secretes the cuticle of the animal from its external (apical) face and supports the somatic nervous system and musculature on its internal (basal) face. The founder cells AB and C generate the entire hypodermis. No

hypodermal deformities were observed in AB(-) mosaics. Similarly, unilateral body muscle mosaics Cap(-) and Cpp(-), but not hypodermal mosaics Caa(-) and Cpa(-), cause observable deformities (described above). Thus, β pat-3 expression in the hypodermis and interfacial epithelia does not appear to be required for embryonic elongation or for normal development. However, one caveat in interpreting these results is that the major hypodermal cell, hyp7, is a syncytium containing over 100 nuclei, and therefore the Ncl marker cannot be used to score the loss of *sDp3*.

Phenotypes of pat-3(rh151)

pat-3(rh151) animals are viable, motile, but non-fertile. The mutation is completely recessive, as demonstrated by the wild type appearance of animals containing *sDp3*. The phenotypes are insensitive to dosage, as *rh151/rh54* animals are identical to *pat-3(rh151)* homozygotes. In addition, the mutation is insensitive to temperature; phenotypes at 15°C and 25°C are identical to those at 20°C.

pat-3(rh151) appears to have defects in a subset of the cells shown in *pat-3(rh54)* mosaics to require β pat-3 function (Table 2). The direct cause of sterility of this allele is not clear; however, the morphogenesis of the somatic gonad is defective. As in *pat-3(rh54)*, the uterine cells and the ovarian sheath cells fail to epithelialize, and the migrations of the ovarian sheath cells are defective. The migrations of CAN, ALM, and the Q descendents are also defective (Fig. 8 and 9). In addition, the outgrowth of the excretory canals is defective in *pat-3(rh151)* mutants. In some individuals, the morphology of the excretory cell is indistinguishable from *pat-3(rh54)* mosaics; however, in most individuals, processes invade both lateral ridges and migrate sufficiently to form a rudimentary H-shape. These canals are short and thick and their channels are invariably swollen and irregular. Phenotypes observed in *pat-3(rh54)* but not in *pat-3(rh151)* include defects in the migration of the sex myoblast, the epithelialization of the distal tip cells, and the attachment of the body wall muscles and other muscles. However, *pat-3(rh151)* body wall muscles may not be attached as strongly as wild type muscles (see Appendix II).

Immunoprecipitations of β pat-3 from pat-3(rh151)

As described above, the *pat-3(rh151)* mutation was shown to result in the conversion of a proline to a leucine. This proline is located in the putative ligand binding region, and

conserved in all integrin β subunits sequenced to date. In addition to a putative role in ligand binding, mutations in this region of vertebrate integrin β subunits have been shown to disrupt integrin subunit association (Arnaout et al., 1990; Wardlaw et al., 1990). To test whether this mutation affects the association of proteins that co-precipitate with β pat-3, the β pat-3 cytoplasmic antibody described in the previous chapter was used to immunoprecipitate β pat-3 from this allele. Compared with stage-matched controls (Fig. 10, lane 3), the level of the proteins co-precipitated with β pat-3 from *pat-3(rh151)* appears to be reduced (Fig. 10, lane 4); this is most noticeable for the 180 kDa and 140 kDa bands. Thus, the *pat-3(rh151)* mutation appears to affect the association of proteins co-precipitated with β pat-3.

Double mutant constructs of pat-3(rh151) with four viable alleles of unc-52

unc-52, which encodes the heparin sulfate proteoglycan perlecan, is a strong candidate for a ligand of β pat-3 for several reasons. First, integrin receptors have been shown to bind to heparin sulfate proteoglycans. Second, Unc-52 staining has been shown to co-localize with the monoclonal antibody MH25 staining in muscle cells during embryogenesis (Hresko et al, 1994), and MH25 was shown in chapter 2 to recognize β pat-3. Third, weak alleles of *unc-52* mutants have defects in body wall muscle cell attachment (Don Moerman, pers. comm.), becoming gradually paralyzed as L4 larvae or adults. Finally, the null phenotype of *unc-52* is Pat (Williams and Waterston, 1994).

Double mutant combination of four viable *unc-52* alleles (*e669*, *e998*, *e1012*, and *e1421*) with the two viable *pat-3* alleles, *rh151* and *rh96* were made. Of the eight possible combinations, only two combinations, *unc-52(e1012); pat-3(rh151)* and *unc-52(e1421);pat-3(rh151)*, had phenotypes different from those observed in either of the single mutants. In these two double mutants, the onset of the Unc phenotype was significantly earlier than in *unc-52* alone. A time course of the onset of the Unc phenotype in the double mutants compared to *unc-52* is shown in Fig. 11. In *unc-52(e1421); pat-3(rh151)*, the time at which 50% of the animals were paralyzed was 35 hours earlier than that observed for *unc-52(e1421)*(Fig. 11A). This difference is especially significant when the timing relative to the developmental stage of the animal is considered: *unc-52(e1421);pat-3(rh151)* animals become paralyzed as late in L2, after the post-deirid has formed, whereas *unc-52(e1421)* animals do not become paralyzed until late L4. In *unc-52(e1012);pat-3(rh151)*, the time at which 50% of the animals were paralyzed was approximately 20 hours earlier than that observed for *unc-52(e1012)* (Fig.

TABLE 2. COMPARISON OF *PAT-3(rh54)* AND *PAT-3(rh151)* PHENOTYPES.

FUNCTIONAL DEFECT OBSERVED	CELLS AFFECTED IN <i>PAT-3 (rh54)</i>	CELLS AFFECTED IN <i>PAT-3 (rh151)</i>
1. Cell attachment.	Body wall muscles. Vulval muscles. Anal depressor muscle.	
2. Cytomorphogenesis.	Somatic gonad. Distal tip cell.	Somatic gonad.
3. Process outgrowth.	Excretory cell. PLM.	Excretory cell. (PLM N/D)
4. Cell migration.	Ovarian sheath cells. Sex mesoblast. ALM, CAN, Q desc.	ALM, CAN, Q desc.

Figure 3-10. Immunoprecipitations of β pat-3 from *pat-3(rh151)* and *pat-3(rh96)*. Immunoprecipitations using the β pat-3 cytoplasmic antibody were performed from protein extracts prepared from equal volume pellets of worms and labeled with biotin. Immunoprecipitates were resuspended in sample buffer, boiled, and loaded on a 6% polyacrylamide gel under non-reducing conditions. The proteins were then transferred to nitrocellulose, the blot incubated with alkaline phosphatase-conjugated streptavidin, and the alkaline phosphatase reaction was used to visualize the proteins. Positions of molecular weight markers, in Daltons $\times 10^3$ are shown to the left. (a) N2 mixed populations. (b) *pat-3(rh96)* mixed populations. (c) N2 L4 larvae and young adults, stage-matched to the *pat-3(rh151)ncl-1* population in (d). *pat-3(rh151)ncl-1* worms were hand-picked by the vulval protrusion phenotype as late L4 larvae or young adults as the *pat-3(rh151)ncl-1* strain is maintained with *sDp3* due to the infertility of these mutants, and thus contains phenotypically wild type animals.

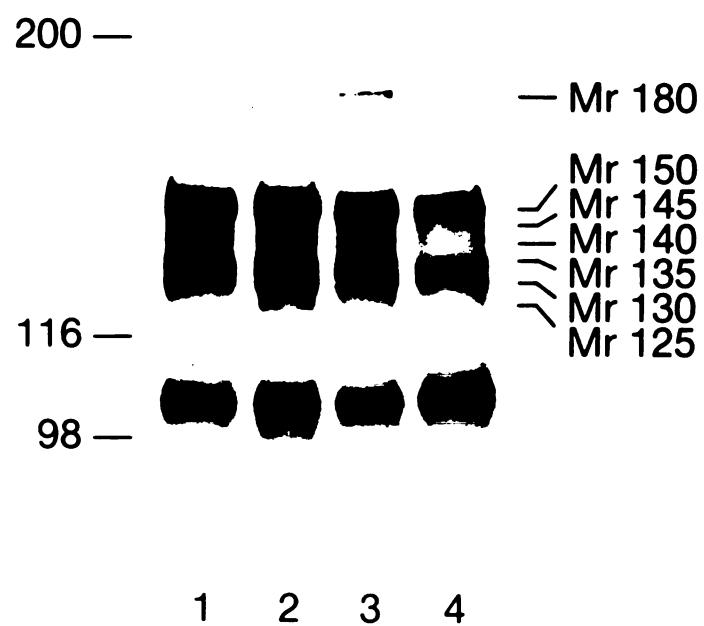
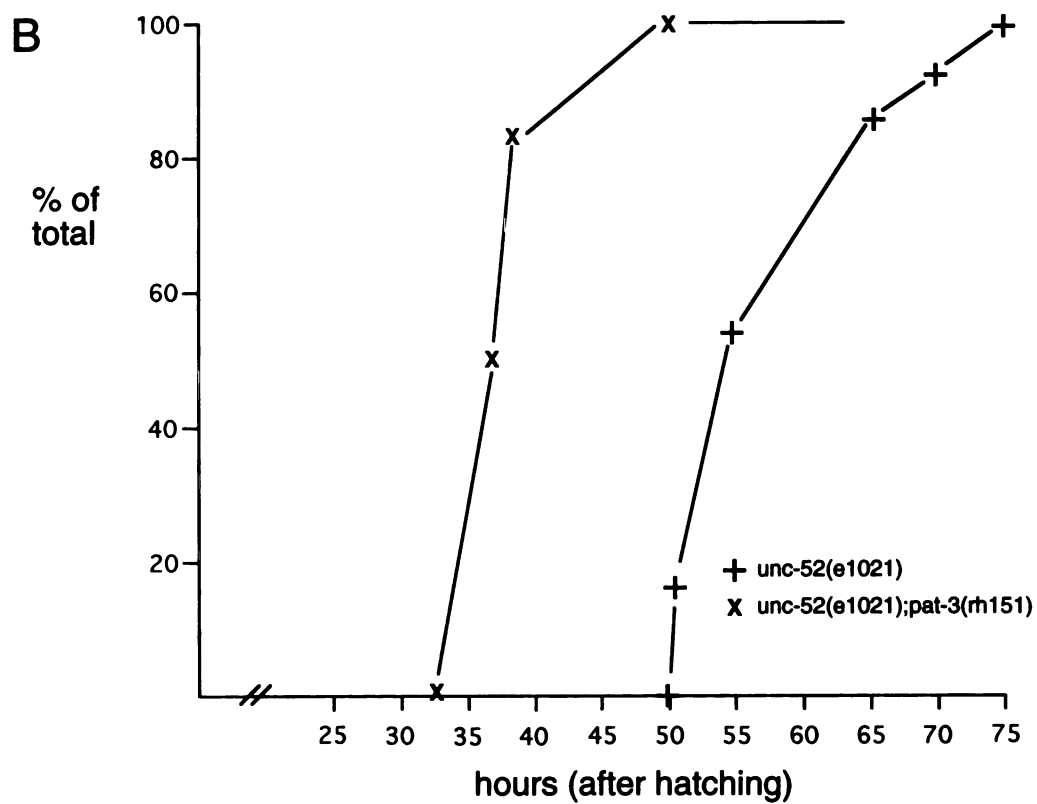
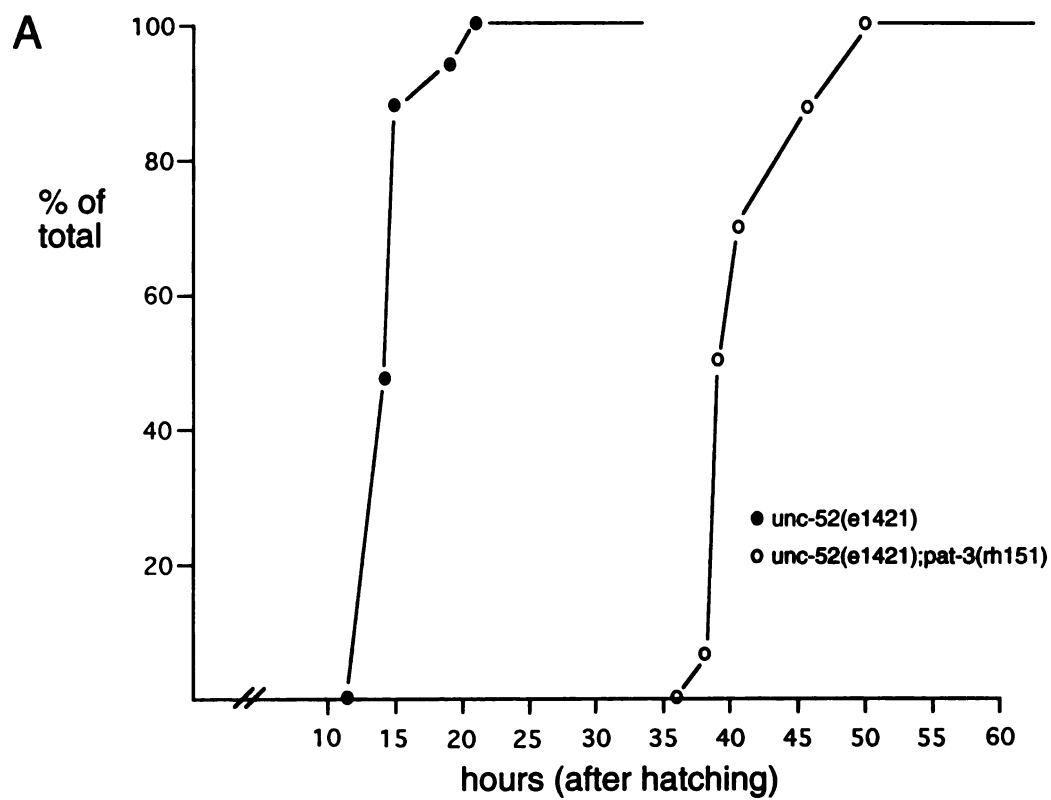


Figure 3-11. Onset of paralysis in *unc-52;pat-3* double mutants. The onset of paralysis after hatching was scored in (a) *unc-52(e1421)* (unfilled dots) and *unc-52(e1421);pat-3(rh151)ncl-1* animals (black dots) and (b) *unc-52(e1012)* (X) and *unc-52(e1012);pat-3(rh151)ncl-1* animals (+). Genotypes and the stage of the animals were verified by DIC microscopy after they were scored as paralyzed by dissecting microscope.



11B). Again, there is a developmentally significant difference in this result: *unc-52(e1012);pat-3(rh151)* animals become paralyzed early in L4, when the vulva is just beginning to form, whereas *unc-52(e1012)* animals become paralyzed as fertile adults. Other observations regarding the double mutants are summarized in Appendix II.

Phenotypes of pat-3(rh96)

pat-3(rh96) is the weakest allele of the three *pat-3* alleles; it is both fertile and motile, with no detectable defects in muscle cell attachment or gonadogenesis. However, subtler versions of phenotypes observed in *pat-3(rh54)* are present. The excretory cell processes invade both ridges and migrate to create well-formed canals of nearly normal length. However, in contrast to wild type animals, in which the excretory canals complete their active outgrowth by the end of the first larval stage, *pat-3(rh96)* canals never quite reach normal length. Correcting for larval growth, we estimate that the posterior canals extend in *pat-3(rh96)* mutants approximately 30% slower than wild type. The migrations of ALM, CAN and the Q descendants are also affected in this allele, but not as strongly as by the *rh151* or *rh54* mutations (Figs. 8 and 9). However, the defect in the HSN migration is more severe than in the null (Fig. 8); the migration stops short posterior to the gonad primordium in L1 larvae. Interestingly, *pat-3(rh96)* has a defect not observed in *pat-3(rh54)* mosaic animals: the migration of the posterior distal tip cell is aberrant in approximately 25% of the animals. This phenotype is variable, ranging from a failure of the gonad arm to reflex back anteriorly, to a looping of the gonad. A comparison of *pat-3(rh96)* phenotypes with *pat-3(rh54)* phenotypes is shown in Table 3.

The molecular analysis of *pat-3(rh96)* revealed that this mutation results in the conversion of an arginine to a cysteine N-terminal to the putative ligand binding region. Immunoprecipitations (Fig. 10, lane 2) and immunoblot analysis (not shown) of β pat-3 from *pat-3(rh96)* animals were performed to see whether the mutation resulted in a misfolded protein having altered mobility on SDS-PAGE. No differences in mobility of β pat-3, or in the pattern of association of co-precipitated proteins was detected.

To test whether the distal tip phenotype in *pat-3(rh96)*, which was not observed in the loss-of-function allele, *pat-3(rh54)*, was due to interference of *rh96* mutant β pat-3 with wild type β pat-3 function (i.e., an antimorph), gene dosage experiments were performed (Fig. 12). In *pat-3(rh96)/pat-3(rh54)* animals, the length of the excretory cell process and the final positions of the Q descendants were more severely affected than in *pat-*

3(rh96) homozygotes. Dosage sensitivity is unique to *pat-3(rh96)*; that is, the wild type integrin gene is not dosage sensitive (*sDp3;rh54* and *sDp3;rh151* animals, which contain one copy of *pat-3(+)* on *sDp3*, are completely wild type). Furthermore, in *sDp3;pat-3(rh96)dpy-17* animals (*dpy-17* was added to mark the chromosome, see methods), the position of the HSN neuron, the length of the excretory cell process, and the posterior distal tip cell migrations are not wild type, although they are less severe than in *pat-3(rh96)* homozygotes (Fig. 12). These results are consistent with *pat-3(rh96)* having antimorphic properties.

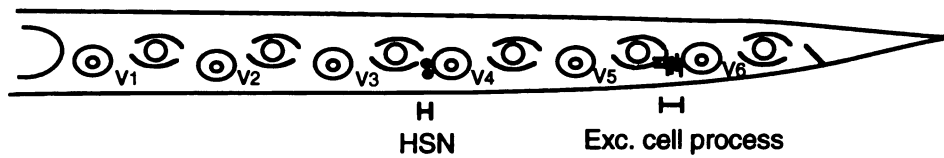
TABLE 3. COMPARISON OF *PAT-3(rh54)* AND *PAT-3(rh96)* PHENOTYPES.

FUNCTIONAL DEFECT OBSERVED	CELLS AFFECTED IN <i>PAT-3 (rh54)</i>	CELLS AFFECTED IN <i>PAT-3 (rh96)</i>
1. Cell attachment.	Body wall muscles. Vulval muscles. Anal depressor muscle.	
2. Cytomorphogenesis.	Somatic gonad. Distal tip cell.	
3. Process outgrowth.	Excretory cell. PLM.	Excretory cell. (PLM N/D)
4. Cell migration.	Ovarian sheath cells. Sex mesoblast. ALM, CAN, Q desc.	ALM, CAN, Q desc. HSN. Post. DTC.

Figure 3-12. The effect of *pat-3(rh96)* gene dosage on the length of the excretory cell canals and HSN final position. The positions of the termination of the excretory cell canals and the final positions of HSN were examined in animals with one copy of the *pat-3(rh96)* mutation (*rh96/rh54*); two copies of the *pat-3(rh96)* mutation (*rh96* homozygotes), and in animals with two copies of the *pat-3(rh96)* mutation and one copy of the wild type integrin gene (*sDp3; rh96dpy-17*). The excretory cell canal and HSN neuron were scored as described in Figures 8 and 9. The HSN neuron is represented by the black dots, and the termination of the excretory cell canal by a T rotated 90° to the right. The anterior of the animal is facing to the left.

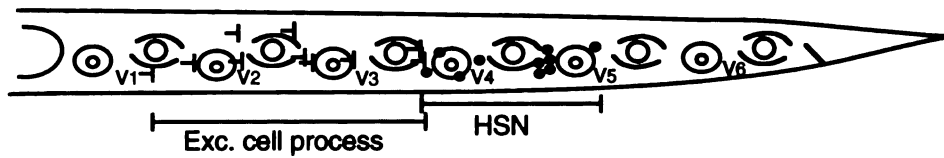
wild type

n=10



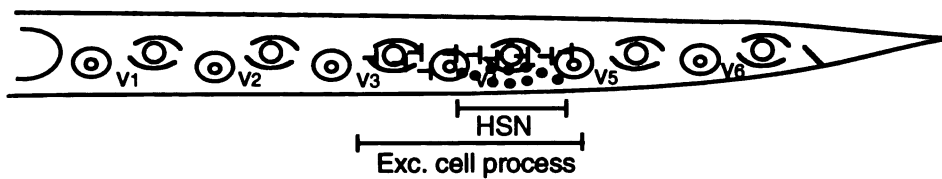
pat-3(rh96)/pat-3(rh54)

n=10



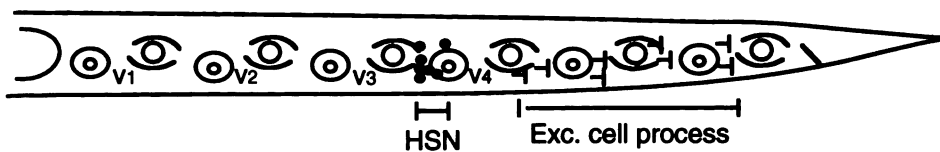
pat-3(rh96)

n=10



sDp3:pat-3(rh96)dpy-17

n=10



DISCUSSION

pat-3 encodes an integrin β subunit

The phenotypes of *pat-3* mutant embryos and mosaic larvae include defects in cell attachment, cell morphogenesis, process outgrowth and cell migration, suggesting that *pat-3* may have a role in cell-matrix interactions (Hedgecock et al., 1987; B. Williams and R. Waterston, pers. comm.). Comparison of the *C. elegans* genetic and physical maps revealed that the integrin β subunit gene described in the previous chapter was a candidate for *pat-3(+)* as both map close to and right of *dpy-27* on chromosome III. We have confirmed that the *pat-3* locus encodes the integrin β subunit gene in two ways. First, both the cosmid ZK1058, which contains the integrin β subunit gene, and a 9 kb Sal-1 fragment containing the complete coding sequence of the integrin gene, complement the *pat-3* null mutation when microinjected into the germplasm of *sDp3;pat-3(rh54)* hermaphrodites. Second, each of three independent *pat-3* alleles induced by ethylmethanesulfonate (EMS) contains a single GC-to-AT transition within the coding sequence of the integrin β subunit gene. Thus, this integrin has been named *C. elegans* integrin β *pat-3*.

β pat-3 is required for the elongation of the embryo

Analysis of the null allele, *pat-3(rh54)*, showed that β pat-3 is not required for early morphogenetic movements in the developing embryo such as gastrulation or neurulation. Furthermore, the early phase of embryonic elongation as well as organogenesis appear to be normal in *pat-3* embryos. In contrast, vertebrate $\beta 1$ integrins have been implicated in early embryonic events such as the spreading of mesodermal cells during gastrulation (Darribere et al., 1988) and the spreading of blastoderm on the vitelline membrane (Lash et al., 1990). Instead, β pat-3 is required for the second phase of elongation of the embryos: while wild type embryos increase to approximately four-fold their original length, *pat-3(rh54)* embryos arrest at the two-fold length. The active forces that produce the elongation of the embryo are thought to be generated by the contraction of radially aligned actin microfilaments in the hypodermis (Priess and Hirsch, 1986). The embryonic sheath is likely to be one source in the transmission of the force generated by the hypodermal cells, as hypodermal constrictions develop in wild type embryos treated externally with trypsin to disrupt the embryonic sheath (Priess and Hirsh, 1986). In addition, it is likely that muscles play a structural role in the transmission of force, as *pat-3* and other mutations that disturb myofibril assembly or contractility also disrupt embryonic

elongation (Priess and Hirsh, 1986; Venolia and Waterston, 1990; Goh and Bogaert, 1991; Williams and Waterston, 1994). Thus, if muscles are not properly attached, the force generated in the hypodermal cells may not be transmitted through the embryo. Consistent with this hypothesis, the localized failure of hypodermal elongation and associated bends, twists, and furrows, are attributable to loss of *pat-3(+)* in clones of underlying body muscles.

β pat-3 is required in mesodermal tissues

β pat-3 has widespread roles in mesodermal development. In skeletal muscles, it is required for muscle attachment to the basement membrane and for the organization of the sarcomeres. Transverse sections analyzed by electron microscopy revealed that thin and thick filaments are present in *pat-3(-)* muscles, although in lower amounts, and can assemble into myofibril lattices; however, mutant muscles do not exhibit birefringence when examined by polarized light, suggesting that the filaments lack wild type organization. This disorganization is most likely due to the lack of dense bodies, which provide anchorage to thin filaments in the longitudinal direction in wild type muscles. As the dense bodies contain vinculin and α -actinin, the implication of this observation is that β pat-3 organizes the dense bodies, which in turn organize the sarcomere. β pat-3 is also required for the attachment of the anal depressor and vulval muscles, and although β pat-3 was detected in the uterine muscles, the intestinal muscles, and the sphincter muscle (see previous chapter), data about the function of β pat-3 in these muscles is not currently available. No role for β pat-3 in the pharyngeal muscles was observed in any of the *pat-3* alleles. However, pharyngeal muscles differ from skeletal muscles in their cytoarchitecture, and contain a pharyngeal-specific form of myosin (Wood, 1988). Possibly, actin filaments in pharyngeal muscles form attachments to the sarcolemma with an integrin distinct from β pat-3 or in an integrin-independent manner.

Development and physiology of the excretory cell

In most animals, renal epithelia have a tubular shape that facilitates molecular sorting as a urine of changing composition moves along the lumen (Riegel, 1972). Both basal and apical surfaces of these epithelia are highly specialized for urine formation. In nematodes, the excretory cell forms a unicellular renal tubule, or protonephridium. Prominent specializations on basal and apical surfaces, i.e., gap junctions with hypodermis and a system of fine canaliculi exist. Like other renal organs, the excretory

cell is probably involved in ionic and osmoregulation, and perhaps waste removal (Nelson and Riddle, 1984). Under favorable conditions, a release of urine through the excretory pore can be observed (Nelson and Riddle, 1984).

In excretory cell *pat-3(-)* mosaics, or *pat-3(rh151)* larvae, the excretory canals are essentially absent. Since the ablation of the excretory cell or its associated duct and pore cells causes a gradual accumulation of water and the eventual death of the animal, it is somewhat surprising that the excretory cell(-) mosaics and *pat-3(rh151)* mutants survive and grow without *bona fide* canals. Residual gap junctions to hypodermis and convoluted tree-shaped channels in the mutant cells evidently provide sufficient renal function for life in laboratory culture. Evidence for the role of integrins in kidney epithelium development has also been observed in vertebrates; antibodies specific for integrin $\alpha 6$ and the E8 domain of the laminin A chain inhibit the formation of tubules and polarization of embryonic metanephric mesenchyme cells (Klein et al., 1988; Sorokin et al., 1990). Interestingly, a laminin A chain homolog has been cloned from *C. elegans*, and a cosmid containing this gene rescues the *epi-1* mutation (Keiichi Joh, pers. comm.). This mutation has defects in excretory cell process outgrowth and gonad epithelialization similar to those seen in *pat-3*.

The nephridia of protozoa and many higher animals have dense networks of small membrane tubules or vesicles at their luminal surfaces that presumably have a major role in secretion or resorption (Patterson, 1980; Zeuthen, 1992). These include the canaliculi in the excretory cell of nematodes. The location, size and morphology of the canaliculi suggest they are related to the plasmalemmal vesicles, or caveolae, observed on many vertebrate cells such as fibroblasts, endothelia, and platelets (see Rothberg et al., 1992). These surface invaginations have a novel striped coat that maintains their characteristic bottle-shape. Caveolin/VIP21, an integral membrane protein, is proposed to be a structural component of this coat (Glenny, 1992; Kurzchalia et al., 1992). The proposed roles of caveolae include transcytosis across endothelia, pinocytosis of small molecules (e.g., folate), and regulation of intracellular calcium. Interestingly, the morphology of the canaliculi in platelets incubated with EDTA changes from tubules to a series of connected vesicles similar to those observed in *pat-3(-)* excretory cells. This phenomenon was shown to be dependent on the presence of the integrin $\alpha \text{IIb}/\beta 3$ receptor, and a differential localization of the subunits within the canaliculi was observed (Gachet et al., 1993).

βpat-3 function in gonadogenesis

In gonadogenesis, βpat-3 is required for the epithelialization of the uterine cells, the ovarian sheath cells, and the distal tip cells. No role for βpat-3 was observed within the germline itself; mitotic proliferation, meiosis, gametogenesis, and fertilization are unaffected in germline *pat-3(-)* mosaics. Interestingly, the migration of the distal tip cell, which secretes a basement membrane as it migrates, is independent of βpat-3, whereas the migration of the ovarian sheath cells, which migrate along the basement membrane secreted by the distal tip cell, is βpat-3-dependent. Instead, the dorsal migration of the distal tip cell is dependent on the products of the *unc-5*, *unc-6* and *unc-40* genes. *unc-5* has been shown to encode a novel cell surface receptor belonging to the Ig superfamily (Leung-Hagesteijn et al., 1992), and *unc-6* encodes a novel protein with some domains homologous to domains IV and VI in laminin (Ishii et al., 1992).

βpat-3 function in the nervous system

Functions of the nervous system including locomotion, feeding, egg-laying, and defecation, are wild type in *pat-3* mosaic animals; thus, it is unlikely that gross defects in connectional patterns result from the loss of βpat-3 function. However, subtle defects in the organization of the nervous system cannot be ruled out until the mutants are serially sectioned and the sections examined by electron microscopy. The lack of βpat-3 function in the development of the nerve cords and ganglia is somewhat surprising, considering the strong expression of the vertebrate integrin β1 subunit in the nervous system, *in vitro* studies showing a role for β1 integrins in neurite outgrowth (reviewed in Reichardt and Tomaselli, 1991), and *in vivo* studies showing a role for β1 integrins in neural crest cell migration (Bronner-Fraser, 1986) and neuroblast migration in the optic tectum (Gallileo, et al., 1992). This apparent conflict can be reconciled if βpat-3 is not considered a homolog of β1, but instead considered a distinct integrin β subunit with properties of integrin β subunits in general (see also discussion, chapter 2). Future studies will reveal whether an integrin β subunit that functions in nervous system development exists in *C. elegans*.

Although βpat-3 has only a limited role in axon guidance, with the sole example detected to date being the outgrowth of the PLM axon, βpat-3 has a more extensive role in neuroblast migration. The embryonic migrations of three pairs of neuroblasts (ALM, CAN, and HSN) and the post-embryonic migrations of four neuroblasts (descendants of the Q neuroblast) are autonomously affected by the *pat-3* mutations. Interestingly, loss of βpat-

3 function in the Q descendants on the right side of the animal results in the termination of the migration approximately one half of the way along the trajectory. Because these neuroblasts are generated from a single progenitor cell and migrate with a stereotyped pattern of cell divisions and programmed cell deaths, it would be of interest to determine whether a particular stage of the migration is affected by β pat-3 loss of function. Unfortunately, this experiment was not done in this study as the loss of function migrations were examined in mosaic animals, which are only rarely isolated.

β pat-3 function in the ectoderm and endoderm

Apart from a limited role in the nervous system, β pat-3 function did not appear to be required in the ectoderm. An unambiguous statement about β pat-3 function in the major hypodermal cell (hyp 7) cannot be made as this cell is syncytial, and a role for β pat-3 in this cell would have been impossible to detect. However, as β pat-3 staining was not detected in the hypodermis, a role for β pat-3 in this cell is unlikely. In addition, no function in *pat-3(-)* intestinal cells was identified. Although β pat-3 staining was detected at the apical surface of the intestine, it was most likely non-specific as intestinal staining was also observed in *pat-3(rh54)* embryos.

Comparison of C. elegans integrin β pat-3 and Drosophila integrin β PS function

The sequence of β pat-3 is most homologous to the *Drosophila* integrin β PS. In both organisms, the integrin β subunits are required for muscle attachment and the organization of the sarcomere. Comparison of the localization of these integrins showed that similarities in the patterns of expression exist: both integrins are present in attachment sites in skeletal muscle and in the visceral muscles that surround the gut. In addition, the β PS integrin is found at the apposed basal faces of the ectodermal and mesodermal sheets during germ band extension (Leptin et al., 1989). In the *Drosophila* integrin β PS mutant, *lethal(1)myospheroid*, germ band extension does not occur. It is possible that germ band extension in *Drosophila* is morphogenetically similar to the elongation of the *C. elegans* embryo. Clearly, it will be interesting to determine if integrin function in these processes are related.

pat-3(rh151) and pat-3(rh96)

A discussion of studies performed with *pat-3(rh151)* and *pat-3(rh96)* is presented in

CHAPTER FOUR

DISCUSSION

The primary objective of this study was to determine whether homologs of known vertebrate adhesion receptors exist in *C. elegans* and if so, to investigate their functions in *C. elegans* development. This objective was met by the cloning and characterization of a novel integrin β subunit at the molecular, protein, and cellular levels. This integrin has been named *C. elegans* integrin β pat-3 as it was shown to be encoded by the *pat-3* locus. With the exception of *Drosophila* integrin β PS, which has limited tissue distribution and functions, the analysis of *C. elegans* integrin β pat-3 has provided an understanding of integrin function during development with cellular resolution not possible in other organisms.

Correspondence of immunocytochemistry and genetics: β pat-3 expression pattern reflects its known functions

In this study, the pattern of β pat-3 expression in developing embryos and larvae was examined by immunocytochemistry and the cells that require β pat-3 function were determined by genetic analysis. How does the pattern of expression correlate with the functional analysis?

In many of the tissues in which a phenotypic defect was observed, β pat-3 expression was detected. In the body wall muscles, β pat-3 localized to cell borders and to dense bodies and M lines, which are the structural anchors for the thin and thick filaments, respectively. In *pat-3(-)* muscles, EM analysis showed that dense bodies did not form and the myofilament lattice was disorganized, suggesting a role for β pat-3 in myofilament lattice assembly via the formation of dense bodies. The results of Hresko, et al. (1994) extend this model with the finding that *unc-52*, which encodes the heparin sulfate proteoglycan perlecan (Rogalski et al., 1993), most likely initiates myofilament lattice assembly via β pat-3. The localization of β pat-3 to the M lines, dense bodies and cell borders in body wall muscles, and to sites of attachment in non-body wall muscles such as the vulval muscles and the anal depressor muscle, suggest a role for β pat-3 in muscle cell attachment. Consistent with this hypothesis, these muscles were found to have defects in attachment. In contrast, the pharyngeal muscles, which did not express detectable levels of β pat-3, were unaffected by β pat-3 loss of function. In the somatic gonad, β pat-3 expression was detected along the outline of the uterus and the spermatheca, but was absent from the germline. Mosaic analysis demonstrated a role for β pat-3 in the epithelialization of the uterus and confirmed a lack of β pat-3 function in gametogenesis and fertilization.

In some cells, β pat-3 function was shown to be required in the cell by mosaic analysis, but β pat-3 expression in these cells was not detected. For example, no staining was observed in the distal tip cell or the ovarian sheath cells, which require β pat-3 for epithelialization. Likewise, β pat-3 staining in the Q and HSN neuroblasts, which require β pat-3 for migration, was not observed. However, as only a few stages of development were examined, staining may not have been detected if the expression of β pat-3 is transient in these cells. For example, the staining observed in the neuron tentatively identified as ALM was observed only during a short time window (approximately 520 minutes to 550 minutes, based on the length of the embryo). Immunocytochemical analysis of carefully staged animals should resolve whether β pat-3 expression can be detected in these cells. β pat-3 expression in the excretory cell, which requires β pat-3 for outgrowth of the excretory canals, could not be convincingly obtained. Occasionally, punctate staining in the approximate location of the excretory cell body and excretory cell canals was detected; however, to verify that this staining is in the excretory cell, double labeling with an antibody that recognizes the excretory cell or immuno-electron microscopy will be necessary.

β pat-3 expression was observed in three tissues in which β pat-3 function was not revealed by mosaic analysis: transient expression in the pharynx in embryos, expression in coelomocytes, and expression at the apical surface of the intestine. The intestinal staining is most likely to be non-specific as it was also observed in *pat-3(-)* embryos. However, staining of the pharynx and the coelomocytes was not observed in *pat-3(-)* embryos, and thus is likely to be dependent on the expression of β pat-3. There are at least two explanations for this apparent discrepancy. First, it is possible that in these tissues, loss of β pat-3 function does not lead to morphologically discernible phenotypes. Alternatively, β pat-3 function could be redundant; that is, other integrins or cell surface adhesion receptors present in the tissues might serve the same function as β pat-3. In chick, outgrowth of ciliary ganglion neurites on cultured Schwann cells is only slightly inhibited by antibodies that block integrin β 1 function, whereas outgrowth is almost completely blocked by a combination of anti-integrin β 1, anti-N-CAM and anti-N-cadherin antibodies (Tomaselli et al., 1988). With the information provided by the immunocytochemical analysis, further genetic studies can be designed to examine β pat-3 function in these tissues.

Finally, neither β pat-3 expression nor β pat-3 function were detected in the nerve cords or in the hypodermis. In contrast, vertebrate integrin β 1 is highly expressed in the

developing nervous system, and *Drosophila* integrin β PS is present in ectodermal cells in embryos (Leptin et al., 1989). These observations support the conclusion, discussed in Chapter 2, that β pat-3 should be considered a distinct integrin β subunit and not a homolog of previously identified integrin β subunits.

Defects of extracellular matrix-dependent cell behavior

Each of the *pat-3* phenotypes is consistent with a defect in cell-extracellular matrix interactions: the coelomocytes, which did not have any discernible phenotypes in the mosaic analysis but strongly express β pat-3, are surrounded by their own basal lamina, myoblasts spread and form specialized muscle attachment sites on the distal face of the hypodermal basal lamina, gonadal cells including the distal tip cells, gonadal mesoblasts, and ovarian sheath cells spread and attach on the proximal face of the gonadal basal lamina, the sex mesoblast and neuroblasts migrate on the proximal face of the hypodermal basal lamina, and the outgrowth of the excretory canals and the PLM axon occur on the proximal face of the hypodermal basal lamina. Indeed, it appears that all of the migrations which are β pat-3-dependent, are also matrix-dependent, whereas at least some, if not all, of the migrations that are β pat-3-independent appear to be matrix-independent. One striking illustration of this point is the migrations of the M mesoblast and its descendent, the sex myoblast (SM). M's migration, which is embryonic and appears to occur independent of a basal lamina, is unaffected by β pat-3 loss of function; however, the migration of SM, which migrates in the second larval stage along a basal lamina, is affected by β pat-3 loss of function.

The interactions of integrins with extracellular matrix proteins have been well documented: cell migration, cell attachment, neurite outgrowth, etc., are mediated by binding of integrins to extracellular matrix proteins (reviewed in Reichardt and Tomaselli, 1991; Hynes, 1992; Hynes and Lander, 1992; Ruoslahti et al., 1993). However, integrins have also been shown to bind to counter-receptors on the surfaces of other cells. Currently, there is no evidence for a role of β pat-3 in cell-cell adhesion; whether counter-receptors will be ligands for other *C. elegans* integrin β subunits, if they exist, or whether the binding of counter-receptor evolved after the split of protostomes from deuterostomes, remains to be seen.

Two other points relating to β pat-3-matrix interactions are worth noting. First, EM analysis showed that the matrix surrounding the muscle is disorganized. Evidence for the

role of integrins in matrix assembly has been provided *in vitro* by Chinese hamster ovary (CHO) cells (Zhang et al., in press) and *in vivo* in *Pleurodeles* (Darribere et al., 1990). Second, in the excretory cell, in intestinal muscle cells, and in the neurons and support cells which form the post-deirid, cell-cell adhesion appears to occur as a default when β pat-3-matrix adhesion is disrupted. The excretory cell makes gap junctions with itself, and the intestinal muscle cells and post-deirid cells, respectively clump together. The significance of the second observation is not apparent at the moment, but it is possible that these cells express molecules such as cadherins, whose function in homotypic cell adhesion is unveiled when cell-extracellular matrix adhesion is prevented.

The role of β pat-3 in cell migration and axon outgrowth: requirement for motility or for navigation?

One of the greatest challenges in the field of developmental neurobiology has been to identify molecules that direct the long-range migrations of neurons and the pathfinding of growth cones. Integrins have been shown to mediate both neuronal migration *in vivo* and neurite outgrowth on a variety of substrates *in vitro* (reviewed in Hynes and Lander, 1992; Tomaselli and Reichardt, 1991). However, the question remains as to whether integrins play a role in the navigation of the cell or axon, or whether they merely participate in the locomotion of the cell or growth cone.

Since β pat-3 plays a minor role in the development of the *C. elegans* nervous system, there is limited data for consideration of this question. However, a couple of observations are worth mentioning. The first observation is with regards to the involvement of β pat-3 in the post-embryonic migrations of the Q neuroblasts. These migrations are asymmetric: the Q progenitor cell is born in the same position on both sides of the animal, but on the right side, the Q descendents migrate anteriorly, and on the left, one of the Q descendents migrates posteriorly. This posteriorly directed migration has been shown to be dependent on the expression of the homeobox gene *mab-5*; in *mab-5(-)* animals, both Q neuroblasts migrate anteriorly (Kenyon, 1986, Salser and Kenyon, 1993). In a *mab-5* gain-of-function allele, or with *mab-5(+)* expression induced by heatshock, both Q neuroblasts migrate posteriorly (Salser and Kenyon, 1992). Thus, Q neuroblasts can migrate in either direction along the body axis. β pat-3 is required for the anterior-ward migration on the right side and the posterior-ward migration on the left. Unless the neuroblasts on the left side use different receptors for anterior-ward migrations than for posteriorly directed migrations, producing a left/right asymmetry in the receptors used for the same

migration, β pat-3 must be capable of mediating migrations in both directions within the same cell. This argument also holds for the posteriorly directed migration of the neuroblasts on the right side. The implication of these observations is that β pat-3 provides the machinery necessary for the migration of the cell, but is not involved in determining the directionality. Further experiments such as determining β pat-3 expression in the migrating Q cells in wild type and in the *mab-5* mutants should provide insight into this question.

The second observation is with regards to the outgrowth of the PLM axon. In this case, it seems that the aberrant migration of *pat-3(-)* PLM axons may be due to lack of β pat-3 function. However, the mosaic animals in which the PLM was mutant also contained other neurons and a few hypodermal cells which were genotypically *pat-3(-)*. Although no function for these cells was observed, a non-autonomous role in disrupting the migration of the PLM axon cannot be ruled out. As this would be the first example of a defect in axonal pathfinding due to loss of integrin function, this finding is worth pursuing.

Is β pat-3 function required for gene expression?

Evidence for the role of integrins in transmitting signals leading to gene expression or cell differentiation is rapidly accumulating. In synovial fibroblasts, metalloprotease genes were induced by fibronectin fragments via $\alpha 5/\beta 1$ (Werb et al., 1989), in melanoma cells, stimulation of $\alpha v/\beta 3$ results in the induction of type IV collagenase expression (Seftor et al., 1992), mesenchyme cells require $\alpha 6$ function for the differentiation of into polarized epithelium (Sorokin et al., 1990), and in monocytes, the expression of immediate early genes such as cytokines and *c-fos* can be induced by cross-linking of $\beta 1$ receptors (Yurochko et al., 1992). Is there any evidence for a role of β pat-3 in regulating gene expression?

Surprisingly, although cytomorphogenesis of some cells did not occur, all of the cells affected by β pat-3 loss of function could be clearly identified. No changes in cell fates were observed. Furthermore, the EM analysis of mutant muscle and the mutant excretory cell showed that cellular specializations of these cells, such as the myofilament lattice and the canaliculi, were present and could assemble within the cell. However, organization of these structures was lacking. Thus, it appears that β pat-3 expression is, to a large extent, not necessary for the expression of proteins which comprise these structures, but is rather necessary for organization of these structures within the cell,

and for the proper differentiated shape of the cell.

Functional consequences of the pat-3(rh151) and pat-3(rh96) mutations

The *pat-3(rh151)* mutation (P203 → L203) is located in a region of integrin β subunits shown by analysis of mutations in the integrin $\beta 1$, $\beta 2$ and $\beta 3$ subunits to be important in ligand binding (Loftus et al., 1990; Takada et al., 1992; Bajt et al., 1992) and in the association of α and β subunits (Kishimoto et al., 1989; Arnaout et al., 1990; Wardlaw et al., 1990; Nelson et al., 1992). *pat-3(rh151)* has defects in a subset of cells that require β pat-3 function. Two possible explanations for this cell-specific phenotype were derived based on integrin biology: First, the mutation could affect the binding of β pat-3 to a subset of its ligands but not to others. This hypothesis was not tested in the present study, but future experiments using purified receptors and ligands could address this question. Alternatively, this mutation could disrupt heterodimer formation with a subset of integrin α subunits with which β pat-3 associates, but permit the dimerization with others. To test this second hypothesis, β pat-3 was immunoprecipitated from *pat-3(rh151)*. The results of the experiment revealed that the level of the co-precipitated proteins was decreased with respect to the level of β pat-3 compared with a control sample prepared from an identical volume of wild type worms. This result is unexpected: assuming that at least some of these co-precipitated proteins represent integrin α subunits, the total ratio of α : β should remain constant since the subunits must dimerize in order to be expressed on the cell surface. However, the biotinylation technique used in this experiment labels intracellular stores of β pat-3 in addition to surface-expressed β pat-3. Thus, undimerized β pat-3 in intracellular stores could be responsible for altering the observed ratio of co-precipitated proteins to β pat-3. If so, this would imply that less receptor is present on the surface of cells. Precedence for this type of mutation exists: a conversion in Leu¹⁴⁹ → Pro¹⁴⁹ in human integrin $\beta 2$ results in greatly reduced cell surface expression of the leukocyte integrins (Wardlaw et al., 1990). Alternatively, the mutation in *pat-3(rh151)* could allow sufficient dimerization of the α and β subunits for cell surface expression, yet interfere with heterodimer association such that receptor function would be disrupted and heterodimer complex stability too weak to withstand the conditions used in the immunoprecipitations. Preliminary examination of β pat-3 staining in *pat-3(rh151)* did not reveal any evidence for a decreased level of expression of β pat-3 in muscle; however, conditions in which β pat-3 expression can be seen in cells affected by the mutation have not yet been defined. The level of cell surface expression of the *rh151* mutant β pat-3 and associated α subunits, when cloned, could be tested in a heterologous system *in vitro*.

The results of the double mutant combinations with *unc-52* suggest that *pat-3(rh151)* may also have a ligand binding defect. This is based on the premature onset of paralysis in only two of the four possible combinations with the four *unc-52* alleles. Furthermore, the double mutants with premature onset of paralysis had phenotypes not seen in either allele alone. It is difficult to account for these observations based on a structural model alone; that is, that reduced surface expression of β pat-3 leads to loss of function in a subset of cells. Moreover, the cells that are defective in *pat-3(rh151)* cannot be grouped by functional category (e.g. some migrations are affected in *pat-3(rh151)*, others that were shown to require β pat-3 by mosaic analysis are wild type; some epithelializations are affected, others are not). Finally, unlike *pat-3(rh54)*, in *pat-3(rh151)*, the excretory cell processes can migrate out to the lateral ridge. These observations can be more easily reconciled by a model that includes a defect in binding to a subset of ligands in addition to a reduction of surface expression of the receptor. Future experiments examining the binding of these receptors to ligands *in vitro* should resolve this question.

The *pat-3(rh96)* mutation results in an additional cysteine C-terminal to the putative ligand binding region (R279 $\rightarrow$ C279). Immunoblot analysis and immunoprecipitations of β pat-3 from *pat-3(rh96)* did not reveal any changes in the mobility of the protein in SDS-PAGE, which suggests that this mutation does not result in the gross misfolding of β pat-3. However, since integrin β subunits have local pairing of cysteines as well as long-range pairing that results in the apposition of linearly distant segments of the protein (Calvete et al., 1991), changes in pairing of cysteines may not result in a protein with altered mobility.

The phenotypes in animals with one copy of the *rh96* mutation (*rh96/rh54*) are more severe than in animals with two copies of the *rh96* mutation. Normally, this allele would be classified as a reduction in function, or hypomorphic, mutation; that is, the function of β pat-3 is dependent on the level of its expression. However, the wild type β pat-3 gene is not dosage-sensitive: one copy of *sDp3* is sufficient to give completely wild type animals. Furthermore, *pat-3(rh96)* has phenotypes not observed in the loss of function mutation, *pat-3(rh54)*. This suggests that *pat-3(rh96)* has properties of an antimorph; that is, *rh96* mutant integrin can interfere with wildtype integrin function to produce novel phenotypes.

An alternative explanation for the increased severity of phenotypes in *rh96/rh54* animals is that the *rh96* mutation results in a percentage of β pat-3 with aberrant function,

and it is the ratio of aberrant to wild type proteins that is dosage sensitive. Interestingly, the length of the excretory cell process, the final position of HSN, and the migration of the posterior distal tip cell are variably affected in *rh96/rh54* animals and *rh96* homozygous animals. Moreover, the excretory process length, the final position of HSN, and the posterior distal tip migration are not wild type in animals containing one copy of the wild type integrin gene with two copies of the *pat-3(rh96)* mutant gene, and the defects are more severe in this strain than in *rh96/+* animals. Thus, the phenotypes observed in *pat-3(rh96)* but not in *pat-3(rh54)* mosaics may be due to the interference of the *rh96* mutant β pat-3 in cells in which loss of β pat-3 function has no effect. Evidence for a dominant negative mutation in the vertebrate $\beta 1$ integrin exists (Takada et al., 1992); a missense mutation (D130 $\rightarrow$ A130) created by site directed mutagenesis abolishes ligand-binding but not heterodimer assembly, and the receptor can be recruited to adhesion plaques. Overexpression at levels two-fold higher than the endogenous $\beta 1$ subunit can result in the inability of the cells to attach to the substrate (Takada et al., 1992). Takada et al. proposed that the dominant negative properties of their mutation could result in a protein that is constitutively in an activated conformation, and therefore competing with wild type receptors for cytoskeletal proteins, but unable to perform its functions due to an inability to bind to its ligand. This model is consistent with what is known about the different conformational states of integrins induced by activation by ligands (reviewed by Phillips et al, 1991; Hynes, 1992; Juliano and Haskill, 1993), by antibody binding (Keizer et al., 1988; Neugebauer and Reichardt, 1991; van Kooyk et al., 1991), or by substances such as phorbol esters (Wright and Silverstein, 1982). This type of conformational change created by mis-pairing of cysteines in a subset of β pat-3 in *rh96* could explain the antimorphic effects observed in *pat-3(rh96)*.

Future directions: developmental genetics of integrin functions in C. elegans

This study has provided understanding of integrin function in *C. elegans* development at the molecular, biochemical and cellular levels. Evidence for the association of β pat-3 with as many as nine proteins with characteristics of integrin α subunits was provided; the identification of these integrin α subunits can be undertaken by using the β pat-3 cytoplasmic antibody to purify these complexes, or by using a genetic approach to identify and characterize mutants with phenotypes similar to *pat-3*. An integrin α gene (F54G8.3) has already been discovered in the genomic sequencing of chromosome III (Sulston et al., 1992), and is likely to encode the *pat-2* mutation (Williams and Waterston, 1994). It remains to be learned whether integrin α F54G8.3 is one of the proteins that

associates with β pat-3.

Likewise, β pat-3 ligands can be identified by multiple approaches. *unc-52* has been demonstrated to interact genetically with *pat-3*, and several other genes for basal lamina proteins have recently been described. These include type IV collagen, laminin and a novel laminin-related protein (Guo and Kramer, 1989; Ishii et al., 1992; Rogalski et al., 1993). Cytoskeletal proteins which may interact with the cytoplasmic domain of β pat-3, such as vinculin and α -actinin have been identified (Francis and Waterston, 1985), and mutations in the vinculin gene exist (Barstead and Waterston, 1991). Suppressor analysis using the *pat-3* mutations may lead to the identification of novel ligands and interacting proteins. By comparing the phenotypes of different mutations that interact with *pat-3*, and combining genetic studies with biochemical studies, a more complete understanding of β pat-3 function may be achieved.

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APPENDIX A

SUMMARY OF DEGENERATE OLIGONUCLEOTIDES USED FOR PCR

Summary of degenerate oligonucleotide primers used for PCR

Gene to be amplified	Name of oligonucleotide	Sequence of oligonucleotide	Predicted size of PCR band	PCR band observed	PCR band sequenced
Cadherin	1. cad-1	5' GG(N)GG(CT)GA(AG)GA(GA)(TC)CA(AG)GA 3'	210	yes	no
	cad-2A	5' TC(AG)TA(N)GGTGG(GT)GC(N)GT(N)GG 3'			
	cad-2B	5' TC(AG)TA(N)GGTGG(CA)GC(N)GT(N)GG 3'			
	2. cad-3	5' GT(N)TT(CT)GA(TC)TA(TC)GA(AG)GG 3'	126	no	
	cad-4	5' TC(N)GC(AG)A(CG)(CT)TT(CT)TT(AG)AA(N)C 3'			
Integrin α subunit	1. FNR α 1	5' GA(TC)GA(TC)AC(TCA)TA(TC)(TCA)T(N)GG(N)TA 3'		yes	yes
	FNR α 2	5' CC(AG)TC(N)CC(AG)T(TC)(N)AC(AG)TC 3'			
Integrin β subunit	1. in β -1	5' GG(N)TT(TC)GA(TC)GC(N)AT(TCA)ATGCA 3'	102	no	
	in β -2	5' (AT)A(AG)TG(AG)AATCC(N)GC(AG)TC(N)GT 3'			
	2. in β -3	5' AT(TCA)CA(TC)GA(TC)CG(TC)CG(TC)GA(AG)TT(TC)G 3'	84	no	
	in β -4	5' TT(AG)TA(AG)TATTGG(AG)TT(TC)TC(N)CC 3'			
	3. β 1#5	5' CA(TC)GA(TC)(CA)GG(CA)G(N)GA(AG)TT(AG)GC 3'	60	no	
	β 1#6	5' GT(AG)TCCCA(TC)TT(N)GC(AG)TTCAT 3'			
	4. β 1#7	5' GA(TC)CT(N)TA(TC)TA(TC)(TC)T(N)ATGGA 3'	135	yes	yes
	β 1#8	5' TC(N)AC(AG)AA(N)GA(N)CC(GA)AA(N)CC 3'			
	cys-1	5' TG(TC)GA(AG)TG(TC)GA(TC)AA(TC)TT 3'	300	yes	no
	cys-2	5' (AG)CACT(CG)(GAC)AC(AG)CA(N)TC(CT)TT 3'			

APPENDIX B

MISCELLANEOUS OBSERVATIONS OF THE *PAT-3* MUTANTS

I. Benthead *pat-3(rh151)* animals.

Rare animals with bent heads identical to the MS(-) losses in *pat-3(rh54)* mosaic animals were observed in plates of *sDp3;pat-3(rh151)ncl-1(e1865)* and *pat-3(rh151)ncl-1(e1865)* animals. Examination by DIC revealed that the dorsal muscles in the head were detached, and no birefringence by polarized light could be observed. The nuclei of these muscles could not be unambiguously identified to verify that they were Ncl, and thus verify that a somatic loss of *sDp3* had occurred; however, neurons in the nerve ring, which are MS derived, were Ncl, and muscles in the region that were attached were non-Ncl. The gonad and excretory cell phenotypes of these animals indicated that these animals were genotypically *sDp3;pat-3(rh151)ncl-1(e1865)* in these cells. These animals were always older; at least L4. One possible explanation for this phenotype would be that *pat-3(rh151)* muscles do not attach as strongly as wildtype muscles, and in older animals, these mutant muscles eventually detach. The benthead phenotype would be more easily observable than losses in other muscles.

II. Other phenotypes of *unc-52;pat-3(rh151)* animals.

e1012:rh151

Homozygous animals were wit; that is, had the withered tail phenotype, which is thought to be due to a defect in the excretory cell. In addition, occasionally in these animals one or two embryos were observed; these embryos did not live. No embryos have been seen in *rh151* animals, and neither animal has the Wit phenotype. *e1012;rh151/+* animals also had an earlier onset of paralysis, although not as early as the double homozygotes. In addition, the heterozygotes had a much reduced fertility, and they were also Wit, but with a later onset.

e1421:rh151

These animals were also Wit.

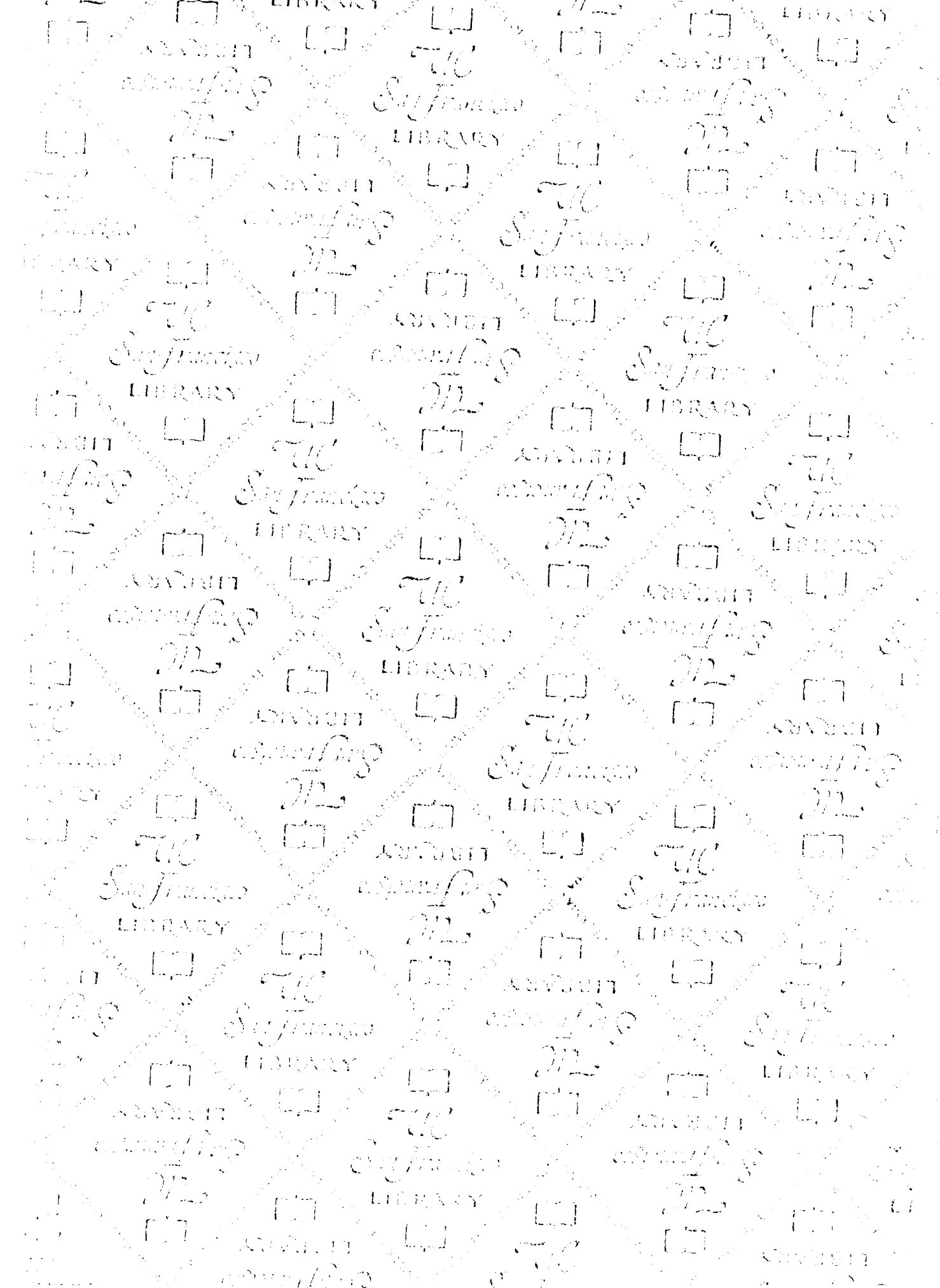
III. *pat-3* male phenotypes.

him-5(e1490) strains of each of the three *pat-3* alleles were constructed.

-*rh54* males will require mosaic analysis to determine all of the phenotypes, and therefore was not examined.

-*rh151* males were examined superficially; defects include crumpled spicules, which may be due to a defect in SM migration, or muscle attachment, defects in ray formation and # of rays produced, and the linker cell often misses the dorsal turn. These animals are likely to be infertile, although this was not tested directly.

-*rh96* males are infertile due to incomplete migration of linker cell; the linker cell reaches approximately the end of the alae, but fails to make the final migration. This migration does not appear to be dosage sensitive (*rh96/rh54* males examined). Most of the male anatomy looks pretty good; however, the gonad is on left side of the animal in 12% of males, as compared with 2% in wild type.



For reference

Not to be taken
from the room.

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