Title
The ornithine decarboxylase deficiency in Caenorhabditis elegans

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Ornithine decarboxylase deficiency in Caenorhabditis elegans: Consequences for embryogenesis

by

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DISSERTATION

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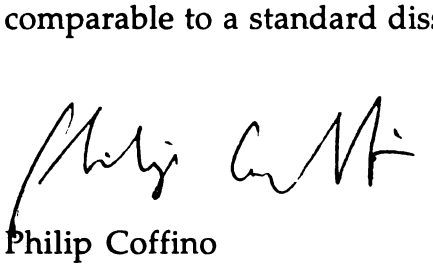
Finally, I thank my family, my husband Donald Macrae, son Cameron and daughter Trisha, for their constant patience and support.

Statement of Declaration

All experiments included in this thesis were performed by Madhu Macrae. She cloned the *odc-1* gene from *C. elegans*. Ronald Plasterk, a co-author of the work included in chapter 1, used her clone to probe a library of Tc1 insertion mutants in *C. elegans*. This insertion mutant of *odc-1*, allele *pk32*, was published by Zwall *et al.* (1994). Madhu found that allele *pk32* was unstable and used this allele to screen for a stable deletion mutant, *pc13*. Most of her thesis work is based on allele *pc13*. She characterized allele *pc13* and developed conditions to study its phenotype. She found *pc13* to be a null allele of *odc-1*. Using this null allele, Madhu was able to show that ODC activity is required for normal development of the worm and that ODC and polyamine deficiency lead to developmental arrest in the worm. This work is comparable to a standard dissertation.



Philip Coffino

ABSTRACT

Ornithine decarboxylase (ODC) is the first enzyme in the biosynthesis of polyamines. Pharmacological studies, the short half-life, and tight regulation of this protein suggested an importance for normal cell growth, but it remained unclear what precise role polyamines, and more specifically ODC, play *in vivo*. I chose *C. elegans* as a model system to investigate the role of ODC *in vivo*.

Degenerate oligonucleotide primers based on sequence homology among mouse, trypanosome and yeast ODC proteins were used to amplify a 150 bp odc sequence from *C. elegans* genomic DNA. This fragment was used as a probe to clone the worm homolog of *odc* cDNA and its gene. Using YAC blot and RFLP analysis, the *odc* gene was mapped to chromosome five between two easily scored genetic markers, *dpy-11* and *unc-42*. In collaboration with Dr. Ronald Plasterk, we obtained an *odc-1::Tc1* mutant *(pk32)* which lacked all detectable ODC enzyme activity due to gene disruption in a conserved region of the protein. These worms display an unexpected wild-type phenotype. To obtain a complete knockout, I isolated a deletion mutant *(pc13)* in which Tc1 had excised from *odc-1::Tc1* imprecisely. *pc13* demonstrated a mild phenotype in regular growth medium, i.e., lowering of progeny number by 35%.

Micro-organisms and cells in culture devoid of ODC activity grow normally if exogenous polyamines are provided. I hypothesized that worms can also utilize polyamines present in the medium, and that deleting polyamines from the growth medium would have an adverse effect on the *odc-1* phenotype. To test this, I screened a series of polyamine analogs to identify those that sustained growth of mutant *E. coli* but could not themselves support the growth of mutant worms. Cadaverine served both these requirements.

Polyamine reduction in the growth medium resulted in two arrest points in worm development depending upon when the restriction was applied. If L1 larvae were transferred to polyamine-deficient medium, they grew up to adult stage with few or no eggs. In contrast, transfer at L4 stage produced progeny arrest during embryogenesis. Nomarski microscopy indicated that, in the absence of polyamines, *odc-1* embryos arrested development at about 550 cells. The two arrest points were specific for ODC deficiency. A wild-type male can partially rescue the embryonic arrest phenotype of the *odc-1* mutant implying that zygotic expression of *odc* is necessary for normal development of the *C. elegans* embryo. However, supplementation with exogenous polyamines can compensate for ODC deficiency in the mutant worm. Mutant males mated with reduced efficiency, even when exogenous polyamines were provided.

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Chapter 1

Introduction

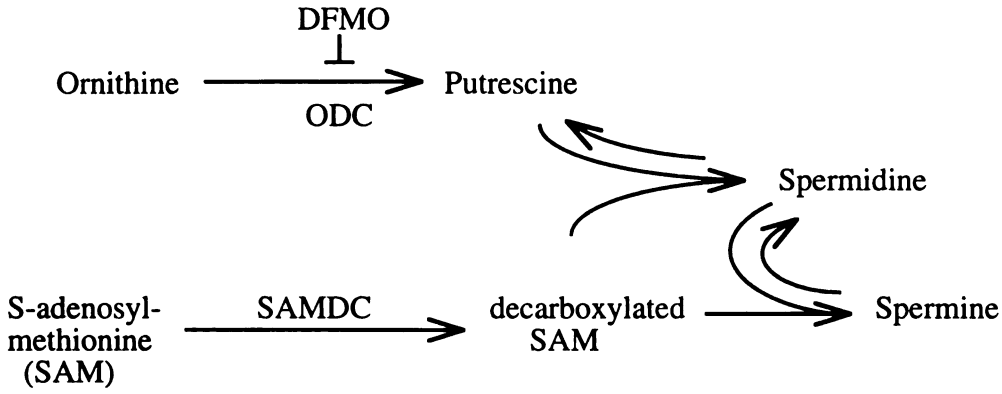
I. POLYAMINES

A. POLYAMINES AND THEIR SIGNIFICANCE

The polyamines, putrescine (a diamine), spermidine and spermine, are constituents of all living organisms examined so far (Pegg 1988). The main source of polyamines in the cell is *via* conversion of ornithine to putrescine, which is catalyzed by the enzyme ornithine decarboxylase (ODC). Putrescine is then converted into spermidine and spermine in a two step process which involves successive transfer of an aminopropyl group from decarboxylated S-adenosyl methionine to putrescine and spermidine, respectively (Figure 1.1). When cells are depleted of polyamines either genetically (Steglich and Scheffler 1982; Tabor *et al.* 1980; Whitney and Morris 1978) or pharmacologically (McConlogue *et al.* 1983), their growth is arrested. Conversely, when polyamines are added to the polyamine depleted cells, they resume their normal growth and differentiation (Steglich and Scheffler 1982; Tabor *et al.* 1980; Whitney and Morris 1978). This suggests that polyamines are important for cell growth. However, the precise molecular mechanism of polyamine function is not understood. Many biological functions for polyamines have been proposed some of which are reviewed below.

a. Polyamines can stabilize nucleic acids: At physiological pH, polyamines are in the protonated form and can carry two, three and four positive charges for putrescine, spermidine and spermine, respectively. In

Figure 1.1 Polyamine biosynthetic pathway



Polyamine biosynthesis: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; SAMDC, S-adenosylmethionine decarboxylase.

the protonated form polyamines can be compared to inorganic cations such as Mg^{++} and Ca^{++}. In contrast to inorganic cations, the positive charge of polyamines is distributed over the entire molecule which may allow flexibility in interaction with macromolecules that are negatively charged such as DNA, RNA or phospholipids (Igarashi *et al.* 1982). Minyat *et al.* (1986) first reported that polyamines may be involved in a conformational change of DNA. X-ray crystallographic data shows that two molecules of spermine bind to tRNA in such a way that the clover leaf structure of the tRNA is stabilized (Quigley *et al.* 1978). Feuerstein *et al.* (1986) extended this study to binding of spermine to DNA. They found that spermine docks in the major groove of B-DNA allowing interactions between the proton donor spermine and the proton acceptor nucleic acid. These interactions help stabilize the nucleic acids. In both models, a 25^{o} bend in the nucleic acid structure was observed to allow docking of the spermine molecule.

Polyamines most likely stabilize the membrane stucture in fragile bacteria (Bacharach 1973; Tabor and Tabor 1964). Grossowicz & Ariel (1963) found that polyamines and other organic cations, but not Mg^{++}, protected the protoplast from lysing. Since very low concentrations of spermine (1 mM spermine as compared to 500 mM sucrose) prevented the protoplast from lysing, Tabor and Tabor (1964) postulated that spermine was not acting via an osmotic mechanism but rather forming complex interactions with the acidic groups in the membrane.

b. Polyamines are important in macromolecular synthesis: When polyamines were depleted from mammalian cells by using inhibitors of polyamine biosynthesis, the rate of ^{3}H thymidine incorporation decreased dramatically. Fillingame *et al.* (1975) concluded that polyamines were important for DNA replication. However, the polyamine analog, methylglyoxal bis-guanylhydrazone (MGBG) used in this study may not be a very specific inhibitor of polyamine biosynthesis. Furthermore, no evidence is available for the interaction of polyamines at the site of replication. Geiger and Morris (1980) used a cell free replication system to examine the importance of polyamines in DNA replication. They reported a 20 fold stimulation in the synthesis of single stranded ϕX174 DNA in a cell free system in *Escherichia coli* (*E. coli*) extracts. Spermidine has also been suggested to control catenation of DNA rings by topoisomerase II (Krasnow and Cozzarelli 1982). Whether spermidine directly affects the activity of the topoisomerase enzyme or the DNA substrate remains to be clarified.

Polyamines may also stimulate transcription reactions. Morris and Hansen (1973) observed a decrease in the elongation rate of β-galactosidase mRNA in auxotrophic *E. coli* when polyamines were removed from the reaction. However, this experiment must be repeated with other mRNAs in order to reach general conclusions about the role of polyamines in transcription elongation.

Tabor and Tabor (1982) demonstrated the requirement of polyamines for efficient translational suppression of amber codons *in vivo*. When a polyamine-deficient mutant of *E. coli*, HT306, was infected with bacteriophage T7 carrying an amber mutation in gene I, it could make only a truncated version of gene I product, even though the bacterial strain contained an amber suppressor. When polyamines were added to this culture, a full length product of gene I was made suggesting that polyamines may be important for efficient translation of amber codons *in vivo*.

A more specific role for spermidine has been defined for the post-translational modification of eIF5A, an eukaryotic initiation factor previously known as eIF4D (Park 1989). eIF5A is an acidic protein of molecular weight 17 kD which is present in all eukaryotes. Although the precise role of eIF5A is not understood, it appears to stimulate the synthesis of methionyl-puromycin in an *in vitro* assay. *In vivo*, expression of at least one of the two eIF5A genes is required for the viability of *Saccharomycese cerevisiae* (Schnier *et al.* 1991, Schwelberger *et al.* 1993). This is further supported by *in vivo* labeling experiment. Park *et al.* (1993) demonstrated that labeled spermidine incorporated into eIF5A and no other protein. In the eIF5A biosynthetic pathway, the 4-aminobutyl group of spermidine is transferred to the ε-amino group of the lysine 50 residue of the eIF5A precursor protein, which is later converted into the mature eIF5A product as the synthesis of the hypusine

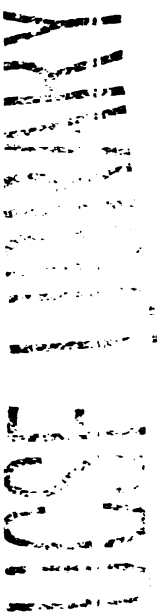
residue is completed. These reactions are catalyzed by the enzymes deoxyhypusine synthase and hydroxylase, respectively.

c. Polyamines may protect the cell from oxidative stress: Oxygen can be very toxic to cells. Anaerobic bacteria are readily killed if allowed to grow in the presence of oxygen. Minton *et al.* (1990) examined the effect of oxygen stress in an *E. coli* strain defective in biosynthesis of spermidine. They found that paraquat toxicity was increased more than ten fold in this strain compared to isogenic strains which can synthesize spermidine. Furthermore, paraquat toxicity was relieved if the medium was supplemented with spermidine. Mutant *spe2 Saccharomyces cerevisiae* (*S. cerevisiae*) cannot synthesize spermidine and spermine. Balasundaram *et al.* (1993) observed growth arrest and cell death in a *spe2* strain of *S. cerevisiae* grown in polyamine deficient medium containing oxygen. In contrast, the growth rate dropped more gradually with no concominant cell death when these cells were grown in polyamine deficient medium either aerobically or anaerobically. They further demonstrated that the *spe2* strain grown aerobically in polyamine deficient medium can not utilize glycerol as a sole carbon source, suggesting that these cells have lost functional mitochondria. The authors concluded that polyamines protect the cell components from oxidative stress but that this is probably not the only function that the polyamines have, since the mutant cells still require polyamines for normal growth even under anaerobic conditions.

d. Polyamines may play a role in apoptosis: The importance of polyamines in biological function is indicated by the normally high intracellular pools of these positively charged molecules (Table 1.1). Prokaryotes have higher intracellular concentration of putrescine than spermidine and spermine (Kashiwagi and Igarashi 1988). Contrary to this, putrescine is immediately converted into spermidine and spermine in eukaryotes, as indicated by higher intracellular concentrations of spermidine than its precursor, putrescine (Eversole *et al.* 1985; Brunton *et al.* 1991). The biosynthetic pathway converts putrescine into spermidine and spermine (Figure 1.1). Acetylation followed by oxidation can reverse this reaction. Acetylation of spermine and spermidine produce N-acetyl spermine and N-acetyl spermidine, respectively. Amine oxidase can metabolize N-acetyl spermidine to produce putrescine and hydrogen peroxide, a byproduct of the reaction, which can be highly toxic to the cells. The higher concentration of spermidine and spermine in eukaryotes compared to putrescine may suggest that putrescine may have a toxic effect on cells. Parchment and Pierce (1989) investigated the role of polyamines in programmed cell death in the mammalian embryo. These authors suggested that catabolism of polyamines by amine oxidase is responsible for programmed cell death that occurs during the formation of the mammalian blastocyst and the limb bud of a 14 day old embryo. The authors observed that malignant cells placed in either of these settings were killed. Inhibitors of amine oxidase (aminoguanidine) and

Table 1.1

	Polyamine Levels		
	Putrescine mM	Spermidine mM	Spermine mM
E. coli (DR112)	28.4	4.2	0
N. crassa	0.32	7.2	0.16
Mammals (BHK21/C13)	0.13	2.07	1.09



Intracellular polyamine content of prokaryotes and eukaryotes.
Reference: Davis, *et al.* (1992)

polyamine toxicity (2-mercaptoethanol) suppressed the killing activity in their experiment. Interestingly, amine oxidase activity has been observed in the mammalian embryo. These studies need to be further substantiated by genetically manipulating the molecules involved.

e. Polyamines may play a role in embryogenesis: Fozard *et al.* (1980) measured the activities of ODC and SAMDC enzymes during mouse embryogenesis. They found that activities of both these enzymes which were generally low until day 5 of gestation in the whole uterus of pregnant mice, rose sharply on days 6 and 7, peaking on day 8 for ODC and day 9 for SAMDC. Although, the increase in ODC activity occured both in embryonic and decidual tissue, the activity was considerably higher in the embryonic tissue. The polyamine levels followed the enzyme activities: putrescine concentration was highest on day 8 of gestation and spermidine on day 9. These authors also examined the effect of inhibition of ODC activity during mouse embryogenesis. Pregnant mice were treated with DL-α-difluoromethylornithine (DFMO, a suicide substrate of ODC) 2% in drinking water during days 5-8 of gestation. The increase in ODC activity on day 8 of gestation and the associated increase in putrescine concentration were both abolished in treated mice. Furthermore, the treated mice did not appear to be pregnant when autopsied on day 18 of gestation. Histological studies indicated that decidualization occured normally after implantation and embryonic development arrested at a stage typical of day 6-7 of gestation in

treated mice. The arrested embryos and the decidual tissue were both subsequently either resorbed or lost from the uterus between days 16-18 (Fozard *et al.* 1980b). The contragestational effects of DFMO were further confirmed in rat and rabbit (Fozard *et al.* 1980b).

B. POLYAMINE BIOSYNTHESIS

The major and direct pathway for the biosynthesis of putrescine is *via* the decarboxylation of ornithine. This reaction is catalyzed by the enzyme ornithine decarboxylase (Figure 1.1). However, in some plants and microorganisms, arginine can also be decarboxylated *via* arginine decarboxylase to produce agmatine, which is later converted into putrescine (Tabor and Tabor 1966b; Morris *et al.* 1970; Roon and Barker 1972). In a separate reaction, S-adenosyl methionine (SAM) is decarboxylated by the enzyme S-adenosyl methionine decarboxylase (SAMDC). Spermidine and spermine are then produced by successive addition to putrescine of an aminopropyl group, donated by decarboxylated S-adenosyl methionine, *via* spermidine synthase and spermine synthase, respectively (Figure 1.1). Although the primary product of putrescine is spermidine, putrescine can also be metabolized in various ways including transamination, oxidation and acetylation to produce γ-aminobutyrate (Seiler and Al-Therib 1974). Hydroxylation and carbamoylation of putrescine to produce 1,4-diaminobutane-2-ol and N-carbamoylputrescine, respectively, are also

possible. Spermidine and spermine are usually the end products in the biosynthesis of polyamines. Spermine can be converted back to spermidine by acetylation followed by oxidation; similarly, spermidine can be converted to putrescine.

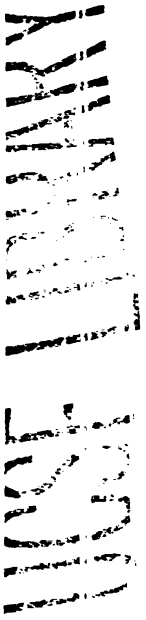
C. ORNITHINE DECARBOXYLASE OF EUKARYOTES

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to produce putrescine. The ODC activity was first discovered in *E. coli* extracts half a century ago by Gale (1946). Since then ODC activity has been identified in all organisms examined so far except in *Trypanosoma cruzi*, a flagellated protozoan that causes intracellular infection in its mammalian host. Although, *T. cruzi* does contain putrescine and spermidine (Bacchi, 1981), attempts to inhibit *in vivo T. cruzi* infection with high levels of DFMO have failed (Hanson *et al.* 1982). However, Kierszenbaum *et al.* (1986) were able to inhibit the mouse peritoneal macrophage infection by blood form of *T. cruzi* with DL- α–difluoromethylarginine, an irreversible inhibitor of arginine decarboxylase, an enzyme that is not found in mammals. Moreover, DFMO did not inhibit the ability of the blood form of *T. cruzi* to infect these macrophages (Kierszenbaum *et al.* 1986). These data suggest that arginine decarboxylase may be the main source of polyamine biosynthesis in *T. cruzi*. Hunter *et al.* (1994) also demonstrated that labeled ornithine cannot be converted into

putrescine in epimastigote stage of *T. cruzi.* However, radiolabeled putrescine is rapidly converted to spermidine and spermine.

In mammals, decarboxylation of ornithine by ornithine decarboxylase is the only pathway for the biosynthesis of putrescine. The mouse ODC protein has a molecular weight of 51,000 daltons for the monomer (McConlogue *et al.* 1985). The protein is active as a dimer and requires pyridoxal phosphate ($PyPO_4$) as a cofactor for its activity. The basal level of ODC activity in quiescent cells is very low. Pegg *et al.* (1982) estimated that each quiescent cell has no more than 200 molecules of ODC. Growth stimuli such as hormones and growth factors can rapidly induce activity of ODC *in vivo* (McCann 1980). The eukaryotic ODC protein which is usually less than 0.0001% of the total soluble protein (Seely *et al.* 1982, Pegg *et al.* 1982, Kameji *et al.* 1982) is too low even after 100 fold stimulation to readily provide protein for biochemical studies (Pegg 1986). Androgen treated mouse kidney cells, the highest source of ODC protein, can make 0.01-0.05% of their soluble proteins as ODC protein. Cells deficient in ODC activity grow poorly (McConlogue *et al.* 1983). McConlogue and Coffino (1983a) selected for overproducers of the ODC protein by treatment of S49 mouse lymphoma cells with DL-α-difluoromethylornithine (DFMO), a specific suicide inhibitor of ODC. This selection amplified the ODC protein more than 300 fold (McConlogue and Coffino 1983a). The DFMO amplified cell line, D4.1, produces 15% of the total cellular protein as ODC (McConlogue and Coffino 1983b). The D4.1 cell line

facilitated the cloning of the mouse ODC cDNA (McConlogue *et al.* 1984). Subsequently, the gene encoding ODC has also been cloned from yeast, *Saccharomyces cerevisiae* (Fonzi and Sypherd 1985); the trypanosome, *Trypanosoma brucei* (Phillips *et al.* 1987); human (Van Steeg *et al.* 1989); and the neurospora, *N. crassa* (Williams *et al.* 1992). Interestingly, in contrast to other eukaryotes, the presence of two genes encoding ODC protein has been reported in the fly, *Drosophila melanogaster* (Rom and Kahana 1993). The gene encoding *Caenorhabditis elegans* (*C. elegans*) ODC is described here (Macrae *et al.* 1995). The deduced protein sequence from all eukaryotic species is highly conserved (Macrae *et al.* 1995). The ODC gene has also been cloned from *Panagrellus redivivus,* also a nematode (Von Besser *et al.* 1995). *P. redivivus*, like *C. elegans*, belongs to the class secernentea. Animals in this class share many similarities in the early embryonic lineages. Details of late embryogenesis, however, have only been studied in *C. elegans*. Postembryonic lineage studies indicate that *P. redivivus* differs in some aspects from *C. elegans* such as cell number, cell fate, *etc.* (Sternberg and Horvitz, 1981, 1982).



Cell and tissue growth has been correlated with increased ODC activity. Russel and Snyder (1968) reported an increase in ODC activity in regenerating rat liver. Normal growth is arrested in cells and microorganisms in which ODC activity has been disrupted either genetically (Tabor *et al.* 1980; Steglich and Scheffler 1982) or pharmacologically (McConlogue *et al.* 1983). Some

growth continues *via* the use of a polyamine analog, cadaverine, generated by decarboxylation of lysine which is one carbon longer than ornithine (Figure 1.2). *E. coli* deficient in the synthesis of polyamines have a growth rate one-third that of wild type *E. coli* (Hafner et al. 1979). Normal growth resumes when polyamines are provided exogenously (Tabor *et al.* 1980). Cohen *et al.* (1978) demonstrated that ODC deficient mutant of *S. cerevisiae* do not sporulate. Mutant Chinese hamster ovary cells, C55.7, do not have a functional ODC gene and cannot synthesize putrescine (Steglich and Scheffler 1982). These cells must be supplemented with putrescine for their normal growth (Steglich and Scheffler 1982).

Higher levels of ODC activity are associated with cell transformation and carcinogenesis. Holtta *et al.* (1993) demonstrated that the higher ODC activity levels are necessary to achieve a tumorigenic phenotype in rat 2R cells infected with a temperature sensitive Rous sarcoma virus. When they added DFMO, a specific inhibitor of ODC activity, to these cells before shifting them to the permissive temperature, the cells did not show a transformed phenotype. Megosh *et al.* (1995) examined the effect of ODC expression in transgenic mice. A construct containing mouse ODC cDNA (-69 to +1682 base pairs of the ODC cDNA) downstream of a bovine keratin IV promoter referred to as K6 minimal promoter (specific for expression in epidermis) was used to generate transgenic mice. Six of eight founder mice had complete hair loss by the second week after birth whereas all F1 progeny showed the

Figure 1.2 Polyamine analogs.

1:4 diaminobutane	(Putrescine)	$NH_2-CH_2-CH_2-CH_2-CH_2-NH_2$
1:5 diaminopentane	(Cadaverine)	$NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH_2$
1:6 diaminohexane		$NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH_2$
1:7 diaminoheptane		$NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH_2$
1:8 diaminooctane		$NH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH_2$

complete hair loss. More interestingly, three of eight founder mice developed visible skin lesions spontaneously. The F1 progeny of the founder mice developed skin tumors at similar frequency, i.e., 40%. The ODC activity in these mice was 11 fold higher than in control mice. No spontaneous tumors were noticed in the control mice. In contrast, Alhonen *et al.* (1995) saw no apparent increase in tumorigenesis in mice overexpressing ODC by 20-50 fold more than the control mice in certain tissues, e.g., brain and kidney. This might be due to the difference in DNA constructs used to generate transgenic mice by the two groups. Alhonen *et al.* (1995) used the entire coding region of ODC (Halmekyto *et al.* 1991) where as Megosh *et al.* (1995) used a truncated form of ODC, which is not susceptible to rapid turnover, to make transgenic mice in the experiments described here.

D. REGULATION OF ORNITHINE DECARBOXYLASE IN EUKARYOTES

The basal enzymatic activity of ornithine decarboxylase is low in all non-proliferating tissues and the activity levels are upregulated more than ten fold in response to growth stimuli (Pegg and Williams-Ashman 1981; Pegg and McCann 1982). Cellular levels of polyamines are tightly controlled. The expression of ornithine decarboxylase and SAM decarboxylase enzymes is highly regulated at transcriptional, translational and post-translational levels in response to growth stimuli.

ODC mRNA is upregulated in Balb/c 3T3 mice in response to growth stimuli such as mitogens, fibroblast growth factor, platelet derived growth factor, and 12-O-tetradecanoylphorbol-13-acetate or TPA (Katz and Kahana 1987). The mechanism of this regulation has been a subject of investigation in many laboratories. Mouse ODC gene contains an enhancer in the first intron of the gene (Chen 1995). There are two six base pair E-box consensus sequences (CACGTG) for binding to the transcription factor myc in this region (Bello-Fernandez *et al.* 1993). These sites are conserved in both rodents and human. When both of these sequences are mutated, the transcriptional regulation of a reporter gene is abolished (Bello-Fernandez *et al.* 1993; Chen 1995). However, another transcription factor with a similar recognition sequence could possibly be involved in the regulation by binding to CACGTG. Therefore, it remains to be determined whether myc is indeed present in this complex. Bello-Fernandez *et al.* (1993) used gel shift assay to further

demonstrate that myc expressed in rabbit reticulocyte lysate associated with the ODC probe (18mer) containing the core wild type sequence (CACGTG) but not the mutant core sequence (CACCTG). This suggested that myc can bind to this sequence and is therefore capable of transactivating the ODC gene. However, whether this is indeed the case *in vivo* remains to be assessed.

Manipulation of cellular levels of polyamines results in rapid changes in the levels of ODC protein. In the presence of polyamines, ODC levels sharply decline. In contrast, when intracellular polyamine levels decline, ODC activity and protein levels rise (Holtta and Pohjanpelto 1986; Kahana and Nathans 1985; Persson *et al.* 1985; Persson *et al.* 1986; Persson *et al.* 1988). Evidence for this conclusion mainly comes from studies of incorporation of ^{35}S methionine in the ODC protein as a measure of protein synthesis. ODC mRNA and activity levels are elevated following treatment of cells with mitogens (Pegg and Williams-Ashman 1981; Pegg and McCann 1982). White *et al.* (1987) observed a discrepancy in stimulation of ODC protein compared to its mRNA when they treated purified bovine lymphocytes with concanavalin A (con A). They argued that efficient translation of the mRNA was possibly responsible for this difference in mRNA and protein levels. In order to prove this, purified lymphocytes from bovine suprapharyngeal lymph nodes were stimulated with 18 μg/ml of Con A. This treatment resulted in a higher recruitment of ODC mRNA into polysomes (White et al. 1987). This suggests that increased efficiency of translation of ODC mRNA

following mitogen activation of lymphocytes is indeed another mechanism for regulating ODC protein levels in the cell.

Cloning of the ODC gene facilitated the study of post-translational regulation of the ODC protein. Mammalian ODC protein has a short half life, 15-45 minutes, depending on the conditions of the experiment (Rosenberg-Hassen *et al.* 1989). In contrast, the wild type ODC protein in *L. donovani*, *L. mexicana*, and *T. brucei* is highly stable and has a half life of more than 6 hours (Parsons and Smith 1989; Hanson *et al.* 1992; Sanchez *et al.* 1989). The post translational regulation of the mammalian ODC protein is at two levels, constitutive and polyamine dependent. Constitutive degradation of the ODC protein is mediated by a sequence inherent to the 37 C-terminal residues of the ODC protein, one rich in PEST amino acids (Rechsteiner 1988; Rogers *et al.* 1986). Ghoda *et al.* (1989) demonstrated that when the 37 amino acids at the C-terminus of mouse ODC protein were deleted, the protein retained full activity and became stable. Furthermore, when they added these 37 amino acids from the mouse ODC protein to the trypanosome ODC protein, the latter, normally a stable protein, became unstable.

ODC protein levels are also negatively regulated by the amount of polyamines in the cell. Ghoda *et al.* (1992) reported that the truncated mouse ODC protein is still subject to polyamine regulation suggesting that this regulation is independent of the constitutive mode of degradation. In mammalian cells, when the intra-cellular levels of polyamines are

augmented, ODC activity goes down about 10-100 fold with a concominant reduction in the amount of ODC protein (Glass and Gerner 1987). However, ODC mRNA levels do not change in response to polyamines. Instead, the reduction in ODC protein results from increased degradation. Its degradation rate correlates well with the amount of a 26,500 dalton protein named antizyme. High levels of polyamines in the cell lead to more efficient translation of the antizyme mRNA by a frame shift mechanism (Rom and Kahana 1994; Matsufuji *et al.* 1995). In turn, antizyme recognizes and binds to the mouse ODC protein (Li & Coffino 1992). The ODC of *T. brucei* is not subject to regulation by high intracellular polyamine levels. Li & Coffino (1992) made a series of enzymatically active chimeras of mouse and trypanosome ODC's in order to determine the antizyme binding and degradation domains in mouse ODC. Replacing residues 117-140 of mouse ODC protein abolished both binding of antizyme and polyamine regulated degradation (Li & Coffino 1992). They further demonstrated that the binding of the C-terminus of antizyme (amino acids 106-212) to the N-terminus of ODC exposed its C-terminus (Li and Coffino 1993). This type of conformational change in the structure of ODC is required for its regulated degradation by the 26S proteosome (Li and Coffino 1994). The role of 26S proteosome in the degradation of ODC has also been reported by Murakami *et al.* (1992).

Polyamines can also be transported into the cell from the growth medium (Tabor and Tabor 1985). The polyamine transport into the cell is precisely regulated based on the intracellular levels of the polyamines. For example, if the intracellular levels of polyamines are high, the polyamine transport pathway is shut down. Although, genes have been characterized for putrescine transport (Tabor and Tabor 1966) and spermidine-putrescine transport (Holtje 1978) in *E. coli*, no mammalian gene for the transport of polyamines has been cloned so far. Polyamine transport mutants have been isolated from mammalian Chinese hamster ovary cells that survive methylglyoxal bis-guanylhydrazone (MGBG, a polyamine analog) toxicity (Mande & Flintoff 1978). Byers *et al.* (1989) complemented polyamine transport deficiency of the mutant Chinese hamster ovary cells by transfecting the cells with DNA from human colon cancer.

The transport system for uptake of polyamines is well established in yeast (Kashiwagi *et al.* 1991; Furuchi *et al.* 1991) and mammalian cells (Mandel and Flintoff 1978; Byers *et al.* 1989). Although the mechanism of transport is not understood, it is clear that when intracellular levels of polyamine levels are depleted by use of ODC inhibitors, increased uptake of polyamines occurs (Alhonen-Hongisto *et al.* 1980; Janne *et al.* 1983). The polyamine transport can be easily modulated by high levels of inorganic cations, Mg++ and Ca++. In the presence of high concentration of Mg++ in the medium, polyamine uptake is inhibited. This suggests that over-

accumulation of polyamines inside the cell can be achieved under magnesium limiting conditions. However, high levels of intracellular polyamines are toxic for the cell indicating that the polyamine uptake mechanism is not down regulated under these conditions (Maruyama *et al.* 1994). Kakinuma *et al* . (1995) used this selection scheme to isolate a polyamine uptake mutant of *S. cerevisiae* that was tolerant to accumulation of spermine in magnesium-limited medium. From the genomic yeast library they isolated a clone (*POT 1)* which rescued the polyamine uptake deficiency. *POT 1* encodes a putative serine-threonine kinase suggesting that polyamine uptake may be regulated by phosphorylation-dephosphorylation.

E. INHIBITORS OF ORNITHINE DECARBOXYLASE

Chemical compounds that can induce polyamine depletion *in vivo* have been used to elucidate the physiological role of polyamines. Both reversible and irreversible inhibitors of polyamine biosynthesis are available. DFMO is a specific irreversible inhibitor of eukaryotic ODC. It binds to the active site of ODC, with a K_i of 40 μM, as a pseudosubstrate and is then decarboxylated (Metcalf *et al.* 1978). Pegg (1986) labeled mouse kidney with [^{14}C] DFMO and analyzed the proteolytic cleavage products of the labeled protein on SDS PAGE gel. Comparison of these fragments with the known sequence of ODC cDNA indicates that DFMO interacts with cysteine 360 which is presumed to be at or near the active site of the ODC protein. Polyamine levels have been shown to peak temporarily during embryogenesis (Fozard *et al.* 1980a). Inhibition of ODC activity by DFMO during mouse gestation led to the suggestion that this activity may be important during embryogenesis. Pregnant mice treated with 2% DFMO in drinking water during days 5-8 of gestation resorbed their foeti (Fozard *et al.* 1980b).

DFMO has been used effectively in the treatment of brain tumors (Marton 1985). DFMO has also been used to treat African sleeping sickness in experimental infections in mice caused by *T. brucei* or human infections caused by *T. gambiense* and *T. rhodesiense*. DFMO inhibits trypanosome ODC activity and therefore depletes the trypanosome polyamines which

results in the inhibition of the growth of bloodstream form of tryanosomes. The basis for DFMO specificity in the treatment of African trypanosomiases is presumably due to the quick depletion of the existing polyamines in trypanosomes as a result of more rapid cell division in this parasite as compared to the mammalian cells (Mamont *et al.* 1976; Porter & Bergeson 1983).

II. THE NEMATODE, *Caenorhabditis elegans*

A. GENERAL FEATURES

Caenorhabditis elegans (*C. elegans*) is a small free living soil nematode. Its short life cycle, small genome size, and ease of genetic manipulation make *C. elegans* a suitable experimental system to study biological problems (Brenner 1974).

In the wild *C. elegans* worms consume bacteria. In the laboratory, they are routinely grown in a petri dish on agar or in liquid culture, using media supplemented with *E. coli*. Mutants are easily obtained by treatment of the worms with conventional mutagens such as ethylmethanesulphonate (EMS). Mutants can be stored indefinitely as L1 larvae at -80°C or in liquid nitrogen. The ease of storage allows generation of large number of mutants that can be characterized when convenient.

C. elegans worms have two sexes, male and hermaphrodite. Progeny are produced either by self fertilization (zygotes produced by union of hermaphrodite eggs and sperm) or by cross fertilization (zygotes produced by union of hermaphrodites eggs and male sperm). Each adult hermaphrodite can lay about 300 eggs. Upon hatching, the animal goes through four larval stages before becoming an adult. The completion of the life cycle takes three days under normal conditions at 20°C.

C. elegans worms are transparent. This feature makes it possible to follow their cell lineage under Nomarski optics. Consequently, position and lineage of each cell has been worked out in this species. An adult worm has about 1000 cells (males have 1031 cells and hermaphrodites have 959 cells).

The haploid genome contains $8x10^7$ base pairs of DNA (Sulston and Brenner 1974). The compact genome size is ideal for genomic sequencing, completion of which is anticipated within the next several years.

B. CLASSIFICATION

The members of the phylum Nematoda are diverse in their habitat. Both parasitic and free living nematodes have a similar body plan characterized by two concentric tubes. The outer tube houses the cuticle, the hypodermis, the muscle, and the nerve cells. The inner tube is the intestine. The space in between the two tubes, the pseudocoelom, contains the gonad. The animals in the genus *Caenorhabditis* are placed in the class Secernentea. The members of this class are terrestrial and free living. *P. redivivus*, like *C. elegans*, belongs to the class secernentea. The animals in this class share many similarities during the early embrogenic lineages, but became dissimilar later in development. The phylogenetic classification is described below:

Phylum	Nematoda
Class	Secernentea
Order	Rhabditida
Family	Rhabditidae
Genus	*Caenorhabditis*
Species	*Caenorhabditis elegans*

C. GENETICS AND LIFE CYCLE OF *C. elegans*

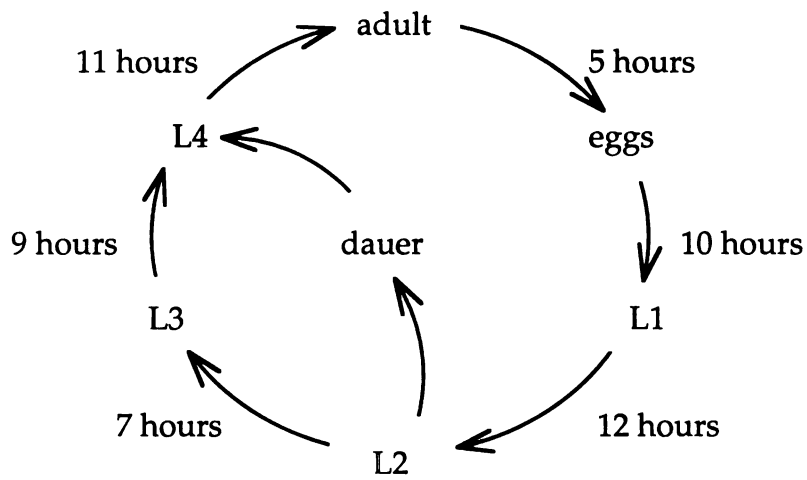
The entire *C. elegans* genome can be mapped to six linkage groups which correspond to the six chromosomes. Each diploid hermaphrodite has five pairs of autosomes and a pair of sex chromosome (XX). The males (XO) are produced by non disjunction of the X-chromosome. This occurs either spontaneously in about 1 of 700 worms or can be induced *via* stress, such as heat shock at 30°C for six hours. Mutants which produce high frequency of males are also available. The haploid worm genome contains $8x10^7$ base pairs and can code for about 5000 genes. This estimate is based on the frequency of lethal mutations and assumptions about the number of essential genes. Less than 20% of the genome is made up of repetitive sequences including transposable elements. The adult hermaphrodite can either self fertilize or mate with a male. If a fertile male is available cross fertilization is preferred. Each adult hermaphrodite can lay 300 eggs by self fertilization under normal conditions. At 22°C eggs hatch 14 hours post fertilization. After hatching the

worm goes through four larval stages, L1 through L4, with each stage punctuated by molts. During the L4 stage, an anchor cell determines the future gonad in the hermaphrodite. The life cycle is complete in 3 days (Figure 1.3). In the absence of food the larva follows an alternate fate at the L2/L3 molt. This specialized L3 larval stage is referred to as the L2D or the dauer stage. The dauer larva stops pharyngeal pumping, develops a specialized cuticle which is impermeable to SDS and can survive dessication for a long period of time. As food becomes available, the larva comes out of dauer stage and enters the life cycle at the L4 stage.

D. EMBRYOGENESIS

In *C. elegans* the process of embryogenesis (from fertilization to hatching) can be divided into three distinct stages: (1) zygote formation and early cleavage, (2) gastrulation and mid cleavage, and (3) morphogenesis. In the first 20 minutes after fertilization, the egg forms a tough shell around itself creating a barrier which is highly impermeable to most solutes, in preparation for survival in the external environment. Cleavage patterns in early embryogenesis of this organism are invariant. In a normally developing embryo, first division is asymmetric and begins 30 minutes after fertilization producing a large blastomere called AB and a small blastomere referred to as P1. The two sister blastomeres have independent fates. They divide at different rates and differentiate into different cell types. For

Figure 1.3 Life cycle of *C. elegans*.



example, only the P1 blastomere progeny can differentiate into intestinal cells. Unequal distribution of determinants in the sister blastomeres is one possible mechanism for the differences in the blastomere fates. Mutations in genes that cause alteration in the normal partitioning pattern of the early embryo have been isolated and characterized. These mutants, *par-1, par-2, par-3* and *par-4* (for partitioning defective), produce developmental arrest during later embryonic stages (Kempheus *et al.* 1988). A series of subsequent asymmetric divisions in the two cell embryo produce six founder cells which are predecessors of the future germ layers of higher eukaryotes (Deppe *et al.* 1978; Sulston *et al.* 1983).

Gastrulation starts at about 100 minutes after fertilization at the 28 cell stage and proceeds until the 200 cell stage. Normally eggs are laid during gastrulation. Cell proliferation and organogenesis continue until about 350 minutes. At about 400 minutes cell proliferation mostly stops and morphogenesis begins. The first twitching movements indicative of muscle cell formation are seen at the 550 cell stage. The embryo at this stage has the appearance of a spheroid. Further development simply leads to elongation of the embryo inside the egg shell without greatly changing its volume or cell number and can be recognized successively as the comma shape, the two fold stage and the three fold stage (also known as the pretzel stage). The three fold stage embryo is highly active in its movements.

Genes whose products are required in early embryogenesis are provided by the mother. The stage at which zygotic transcription begins is not invariant in all organisms (Davidson 1986). In *C. elegans*, embryonically transcribed mRNAs have been detected at the 8-12 cell stage using incorporation of ^{32}P UTP into TCA precipitable material and autoradiography of ^{3}H UTP labeled embryos in the presence and absence of α-amanitin (*Edgar et al.* 1994). Using in-situ hybridization, Seydoux and Fire (1994) reported the appearance of embryonic transcription at the 4-cell stage.

At 800 minutes after fertilization, the embryo hatches. Under normal conditions, the newly hatched larva begins to feed and grow. Further cell divisions occur and the rest of the somatic cell and germ cell lineages are completed during larval stages L1 through L4. The mature adult has about 1000 cells.

E. TARGETED GENE DISRUPTION

Isolating a null mutant in a gene of interest facilitates investigation of the function of that gene. In yeast and mammals, homologous recombination is used to disrupt a gene. Transposon tagging has been used for that purpose in drosophila and has recently been applied to *C. elegans*. There are at least 8 types of transposons found in *C. elegans*, of which Tc1 is the most studied. The wild type Bristol strain contains a few copies of the transposon Tc1. In contrast, the mutator strain Bergerac contains hundreds of

copies of the Tc1 transposon. Tc1 is 1.6 kb in length, has an inverted repeat at the ends of the transposon and codes for its own transposase. Tc1 is more similar to the bacterial IS1 transposon than the eukaryotic transposons such as the P-element of flies. Zwaal *et al.* (1993) have identified a number of mutants for disruption of cloned genes by screening a frozen library of the Bergerac strain. In order to screen for a Tc1 insertion in the gene of interest, they first amplified the DNA around the insertion site of Tc1 by using one primer within the Tc1 sequence and a second primer in the sequence of interest. The size of the amplified product is identified by gel electrophoresis and the site of transposon insertion is determined by sequence analysis. Once the Tc1 insertion mutant is identified, a mutant containing a stable partial deletion of the Tc1 gene from the germ cells is isolated in a screen using PCR amplification. Using this two step process it has been possible to obtain deletions within cloned genes.

III. RESEARCH AIMS

Numerous studies have indicated that polyamines are needed for optimal cell growth, but no specific role has been assigned to them *in vivo*. Activity of ornithine decarboxylase, the first enzyme in the biosynthesis of polyamines, is highly regulated at transcriptional, translational and post-translational levels. Deficiency of intracellular ODC protein can be overcome in cultured cells (Chinese hamster ovary cells lacking ODC activity) and micro-organisms (polyamine auxotrophic yeast, fungi and bacteria) simply by supplying polyamines in the medium in which the cells are grown. The focus of my research is to elucidate the principal role of polyamines in growth and development of a multicellular organism which is biologically more complex than single cell organisms such as yeast and bacteria. *C. elegans* is an excellent choice as it allows the use of molecular and genetic approaches together in order to address biological questions. The species *C. elegans* belongs to the phylum Nematoda. Organisms of this phylum are diverse and provide many advantages and disadvantages to humans. For example, worms that live in the soil can provide aeration to the soil while keeping the bacterial contamination in check. Conversely, some species can do a great deal of damage to crops. As parasites, nematodes are also responsible for causing many diseases, especially in children. Understanding the role of *C.*

elegans ODC will be helpful in identifying ways to treat nematode related problems in the future.

Toward fulfilling this goal, I report the cloning and characteristics of the *C. elegans* ODC gene in chapter one. I show data that suggests a role of ODC activity during larval growth of the worm. However, gene disruption of ODC by transposon tagging does not produce a significant alteration in phenotype of the mutant worms in normal growth medium. Homozygous mutant hermaphrodites produce 36% fewer progeny (chapter two) and mutant males mate inefficiently compared to their wild type counterparts (chapter three) in normal growth medium. In chapter three, I also describe the consequence of depleting the growth medium of polyamines on the *odc-1* mutant. The data shows two specific arrest points in *C. elegans* development. Depending upon the larval stage at which the polyamines are removed from the growth medium, the *odc-1* mutant worms either grow up to be sterile adults or show developmental arrest at the embryonic stage. However, these phenotypes are not completely penetrant. A wild type male can rescue the embryonic arrest of the mutant worm suggesting that zygotic expression of ODC gene is required for normal development of the *C. elegans* embryo in polyamine deficient medium. However, this function can be compensated simply by supplying polyamines exogenously in the growth medium.

Chapter 2

The ornithine decarboxylase gene of *Caenorhabditis elegans*: Cloning, mapping and mutagenesis

ABSTRACT

The gene *(odc-1)* encoding ornithine decarboxylase, a key enzyme in polyamine biosynthesis, was cloned and characterized. Two introns interrupt the coding sequence of the gene. The deduced protein contains 422 amino acids and is homologous to ornithine decarboxylase of other eukaryotic species. *In vitro* translation of a transcript of the cDNA yielded an enzymatically active product. The mRNA is 1.5 kb in size and is formed by trans-splicing to SL1, a common 5′ RNA segment. *odc-1* maps to the middle of LG V, between *dpy-11* and *unc-42* and near a breakpoint of the *nDF32* deficiency strain. Enzymatic activity is low in starved stage 1 (L1) larva and, after feeding, rises progressively as the worms develop. Targeted gene disruption was used to create a null allele. Homozygous mutants are normally viable and show no apparent defects, with the exception of a somewhat reduced brood size. *In vitro* assays for ornithine decarboxylase activity, however, show no detectable enzymatic activity, suggesting that ornithine decarboxylase is dispensable for nematode growth in the laboratory.

INTRODUCTION

Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthetic pathway. Polyamines are essential for cell growth (Tabor and Tabor 1984). Mutant Chinese hamster ovary cells lacking ODC activity do not grow in polyamine-free medium, but grow normally if supplemented with putrescine, the product of ODC catalysis (Steglich and Scheffler 1982; Pohjanpelto *et al.* 1985). More generally, depleting cells of polyamines by genetic or pharmacological means has detrimental effects on growth and survival, but these harmful effects can be prevented by providing an exogenous source of polyamines (Davis *et al.* 1992). In mammalian cells, ODC has a short half life (Russel and Snyder 1969) and is highly regulated (Pegg 1986). Understanding the mechanisms of regulation of ODC has been facilitated by cloning the gene from human (Van Steeg *et al.* 1989), mouse (Gupta and Coffino 1985), trypanosome (Phillips *et al.* 1987), Leishmania (Hanson *et al.* 1992), yeast (Fonzi and Sypherd 1985), Neurospora (Williams *et al.* 1992) and Drosophila (Rom and Kahana 1993). Both transcriptional and post-transcriptional mechanisms have been shown to be important in controlling the enzyme. The elaborate regulation of ODC in eukaryotes, elicited largely in response to signals for growth or differentiation, lacks a clearly defined role, despite a welter of descriptive information. One approach to addressing this problem lies in using an organism in which ODC

can readily be subjected to genetic manipulation, and in which differentiation has been well studied.

To investigate the role ODC enzyme plays in development, we chose the small free living nematode *Caenorhabditis elegans*. This organism is multicellular, has a short generation time of ~3 days and is readily manipulated in the laboratory (Wood *et al.* 1988). It has a small genome size, and its cell lineage, which is invariant among individual animals, has been completely worked out (Sulston *et al.* 1983), making it an excellent model for genetic analysis of development. We report here the cloning and characterization of the *C. elegans odc-1* gene and its cDNA. We have also isolated and partially characterized an *odc-1* null mutant.

MATERIALS AND METHODS

Cloning and sequencing of *C. elegans odc-1*

Highly degenerate primers corresponding to the conserved region of mouse ODC residues 64-70 (5′ GGGAATTCCTTYTAYGCNGTNAARTG) and residues 110-115 (5′ GGGGATCCCTTRCANGGRTTNGCRTA) with EcoRI and BamHI sites, respectively, at their 5′ ends were used to generate an ~150-nt PCR fragment. The PCR reaction was performed with Taq polymerase (Perkin Elmer Cetus Corp.) using the buffer conditions recommended by the manufacturer. Briefly, the reaction was carried out for 35 cycles at 94^0 for 30 sec, 37^0 for 1 min, and 72^0 for 1 min. The 150-nt PCR fragment was then cloned into the EcoRI-BamHI sites of the vector pTZ18u (USB). The recombinant (pC5) was sequenced to confirm the identity of the clone. This clone was used to probe lambda recombinant libraries contaning *C. elegans* sequences, either genomic, in the lambda 2001 vector, or cDNA, in the lambda SHLX2 vector (Palazzolo *et al.* 1990). Genomic and cDNA libraries were kindly provided by Sasha Kamb and C. H. Martin, respectively. Genomic and cDNA clones were sequenced by the dideoxy sequence method of Sanger using the Sequenase II kit, purchased from USB. The sequences have been reported to GenBank (Accession No. U03059).

Restriction mapping of cloned *odc-1* DNA

The recombinant inserts cloned in lambda vectors were restriction mapped by partial digestion as described in Rackwitz *et al.* (1984), with some modifications as follows. Lambda clones harboring *odc-1* were partially digested with the restriction enzymes HindIII, EcoRI, SstI, XbaI or ClaI, or with pairs of these enzymes and resolved on a 0.7% agarose gel. The DNA fragments were transferred to a nylon membrane and then probed with end-labeled primers homologous to lambda arms λ-ON-R and λ-ON-L (Rackwitz *et al.* 1984). The size of labeled bands was inferred from electrophoretic mobility, using as a calibration standard a 1-kb DNA ladder (BRL).

Isolation of an *odc-1* null mutant strain

Tc1 transposon insertion within *odc-1* was screened by PCR in MT3126 strain with a combination of *odc-1* and Tc1 specific primers:
(*ODC primers* 3174: 5′ TACGATCCTTTGCGTGCAGGAG 3′
and primer 3468: 5′ TGTAGTACATGTATCCATGATC 3′;
Tc1 primers L1: 5′ TGTTCGAAGCCAGCTACAATGGC 3′ and
L2: 5′ TCAAGTCAAATGGATGCTTGAG 3′) as described in Zwaal et al. (1993). Among the progeny of one such *odc-1::Tc1* worm, *odc-1 (pk32)* (Zwaal *et al.* 1993), further screening yielded a mutant with a partial deletion of the Tc1 element, *odc-1* allele *pc13*. This mutant was outcrossed 10 times to the N2

wild-type Bristol strain, and the resultant strain carrying *pc13* was designated SF1.

Genetic and physical mapping of *odc-1*

The *C. elegans* Yac blot (Coulson *et al.* 1991, obtained from the laboratory of Robert H. Waterston at the Washington School of Medicine, St. Louis, MO) was hybridized with a ^{32}P-labeled probe prepared by random priming of the *odc-1* cDNA clone, washed and subjected to autoradiography. To further localize the position of *odc-1* with respect to a deficency in the region surrounding *dpy-11*, genomic DNA from either wild-type or the *nDf32* deficiency strain, genotype [*nDf32*(V) *eT1*(III;V) *unc36*(III)], obtained from the *Caenorhabditis* Genetics Center, Univ. Minnesota, was restricted by Eco RI, Pst I, Xba I or Hind III, or by combinations thereof, and subjected to Southern blotting, as described below. To localize *odc-1* on the genetic map by three point recombination, the progeny of *odc-1(pc13)/dpy-11(e224) unc-42(e270)* heterozygotes were screened for Dpy non Unc or Unc non Dpy. The recombinants were analyzed for the presence of *pc13* by PCR.

Growth of worms and synchronization of developmental stages

The N2 strain of *C. elegans* was grown and synchronized with respect to developmental stage as described (WOOD *et al.* 1988; Land *et al.* 1994). Briefly, gravid adults were washed from plates with M9 buffer and bleached

(worms:M9:4-6% NaOCl:1 N NaOH; 1:1:2:2 by volume) with frequent mixing at room temperature for 4-5 min or until no adult carcasses remained intact. Eggs were immediately washed several times in M9 buffer and allowed to hatch over-night either on unseeded NG plates or S-medium without *Escherichia coli*. A portion of L1 larvae hatched without *E. coli* as a food source was harvested and the remainder suspended in S-medium containing *E. coli* and periodically harvested.

Assay of ODC enzyme activity

For measurement of ODC activity, worms were fed with *E. coli* strain HT 289 (*speA*$^-$, *speB*$^-$ and *speC*$^-$), which is deficient in ODC and other polyamine biosynthetic enzymes (Tabor *et al.* 1981). The worms were harvested and sonicated for 20 times 1 sec in assay buffer (40 mM KPO4, pH 7.5, 0.1 mM pyridoxal phosphate and 2 mM DTT). The lysate was spun for 10 min. at 4^0 C in a microfuge and the enzyme activity in the supernatant was measured as described (Li and Coffino 1992). Briefly, 0.1 ml of the extract was incubated for 15 min in the presence or absence of 0.1 mM difluoromethyl ornithine (DFMO), a specific mechanism-based inhibitor of ODC (Metcalf *et al.* 1978). Activity was assayed in the presence of 0.2 mM L-ornithine, 1 μCi of ^{14}C ornithine (specific activity, 50 mCi/mmole, purchased from NEN) at 37^{o}C for one hour. Labeled CO_2 evolved by enzymatic decarboxylation was

trapped with hyamine hydroxide and counted in a scintillation counter. Protein was measured by Lowry assay (Lowry *et al.* 1951) and enzyme activity expressed as nmoles $^{14}CO_2$ per hour per milligram of lysate protein.

Transcription and translation of cloned *C. elegans* odc was carried out *in vitro* as described (Li and Coffino 1992), with the following changes. The cDNA clone harboring the coding region of *odc-1*, pCEC1a, was linearized with ApaI (located at the 3' end of *odc-1*), transcribed with SP6 polymerase and translated using the Promega's rabbit reticulocyte lysate kit. When the enzymatic activity of the *in vitro* translation product was to be determined, radiolabeled methionine was replaced by unlabeled methionine.

Southern and Northern Blots

C. elegans genomic DNA or lambda clones harboring genomic *odc-1* were digested with restriction enzymes, purchased from either New England Biolabs or BRL, using the conditions described by the manufacturer. The restricted DNA was resolved on a 0.7% agarose gel in 0.5X TBE, denatured and transferred to HYBOND-N (Amersham) in 20X SSC over night. The filters were probed with *odc-1* cDNA restriction fragments that were ^{32}P-labeled by random priming.

To isolate RNA, a mixed population of worms was harvested from one 50 mm petri dish. Total RNA was prepared according to the protocol of Promega Biological Research Products, except that the worms were broken by

vortexing for 4 min. in the presence of sand. Where indicated, polyA$^+$ mRNA was isolated using an oligo dT column purchased from Stratagene. A 10 µg sample of total RNA or 2 µg of polyA$^+$ mRNA was resolved on a 1% agarose gel containing formaldehyde. The RNA was transferred to a nylon membrane and hybridized to the *odc-1* probe as described for Southern analysis.

Reverse transcription-PCR

One microgram of total RNA from eggs or mixed population of worms was reverse transcribed with MMLV reverse transcriptase (Promega Scientific) at 42°C for 2 hours according to the protocol provided by the supplier, using ce66rc primer (5' terminus positioned at codon 66 of the *C. elegans odc-1* open reading frame, with its orientation that of reverse complement). The RT reaction included 50 mM Tris pH 8.3, 60 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM dNTPs, 2 units RNasin, 25 pmoles ce66rc primer, and 20 units MMLV reverse transcriptase in a 10 µl reaction. The cDNA was amplified with Taq Polymerase in the same buffer using either an SL1 (GGTTTAATTACCCAAGTTTG) or SL2 (GGTTTTAACCCAGTTACTCA) splice leader primer (Nilsen 1993) and ce66rc, an *odc* specific primer. The reaction was carried out for 30 cycles at 92°C for 1 min, 55°C for 1 min, and 72°C for 2 min in the MJR thermocycler. A small aliquot of the PCR product

was resolved on a 1% agarose gel, transferred to a nylon membrane and probed with the 5' fragment of *odc-1*.

RESULTS

Cloning and sequencing of the *C. elegans odc* gene and cDNA

PCR amplification of *C. elegans* genomic DNA with degenerate primers corresponding to the highly conserved sequence of mouse, trypanosome and yeast ODC produced a 150 nt fragment which had a unique nucleotide sequence but a deduced amino acid sequence homologous to other ODC proteins (M.A. Phillips, M. Macrae., C. B. Stewart and P. Coffino, unpublished data). The region of homology corresponded to amino acids 64-115 of mouse ODC (results not shown), confirming that the PCR produced the anticipated product fragment. This fragment was used as a probe to screen genomic and cDNA libraries from the N2 strain of *C. elegans*. Screening a lambda genomic library revealed four positive clones among approximately 18,000 plaques. Southern analysis with ClaI restriction endonuclease showed one hybridization pattern for genomic clones 1 and 3 and another for clones 2 and 4. On the other hand, analysis with SacI, XhoI and XbaI restriction endonucleases gave an identical pattern for all four clones. We picked genomic clone 2 for further characterization since it gave an identical Southern blot restriction pattern as the genomic DNA with all enzymes tested.

To fully characterize the open reading frame and to provide a ready source of probes for further analysis of the genomic clones, we used the same

150 nt PCR fragment as a probe to isolate cDNA clones. The sequence of such a cDNA contained one long open reading frame encoding 422 amino acids, followed by an OPAL stop codon and an AATAA poly A signal 27 nt 3' to the stop codon. The *odc-1* cDNA was transcribed and translated *in vitro* and ODC enzyme activity so generated was measured as described (Li and Coffino 1992). The activity was similar in quantity to that seen when trypanosome *odc* DNA was subjected to *in vitro* transcription and translation in parallel and, like other eukaryotic ODCs previously examined, was inhibited by alpha-difluoromethyl ornithine (DFMO), a mechanism-based suicide-substrate inhibitor (Metcalf *et al.* 1978) (results not shown). This confirms that the cloned *C. elegans* cDNA encodes authentic and functional ODC. The deduced protein has strong homology to other eukaryotic ODCs. The sequence of genomic *odc-1* clone 2 was found to be identical to that of the cDNA, except that the gene has two introns 57 nt and 47 nt long which interrupt the coding region of *odc-1*, the first within amino acid codon 152 and the second after codon 253 (Figure 2.1).

Figure 2.1. Sequence of wild-type and mutant *(pc13)odc-1* gene and protein. (A) Sequence of *odc-1* gene and cDNA and inferred ODC protein. Upper case nucleotides: sequence of cDNA corresponding to the deduced open reading frame. Lower case: introns, untranslated 5' and 3' flanking regions. The sequence of SL1 (lower case, underlined) is shown and the site of trans-splicing indicated. Broken arrows show the position of the degenerate PCR primer pair used to generate a probe specific for *odc-1*. The solid arrow marks the position of the internal primer used with an SL1-specific primer for RT-PCR. Tc1 insertion site, a TA dinucleotide, is underlined. (B) Sequence of the 112 nucleotide 3' Tc1 remnant and contiguous *odc-1* sequence in *pc13*. Numbers refer to amino acid position in wild-type ODC. Deletion of a single nucleotide in *pc13* has converted CGT arg (109) to Cat (his). Above, nucleotide sequence, where upper case represents sequence derived from *odc-1* and lower case that derived from Tc1. Below, Translation to the three-letter amino acid code. (C) Comparison of amino acid sequences of ODC of different species in the region of the Tc1 insertion. The Tc1 encoded amino acid insert sequence in the *pc13* allele is represented as "+37", and corresponds to the sequence shown in Figure 1B. Species and GenBank accession numbers are as follows: mouse *(Mus musculus)* No. M10624; frog *(Xenopus laevis)* No. X56316; chicken *(Gallus gallus)* No. X64710; Trypanosome *(Trypanosoma brucei)* No. J02771; Drosophila *(Drosophila melanogaster)* No. X66601; yeast

(Saccharomyces cerevisiae) No. J02777; Leishmania *(Leishmania donovani)* No. M81192; and Neurospora *(Neurospora crassa)* No. M68969.

Figure 2.1A Sequence of *odc-1* gene and protein.

```
                                        250
TTT CCA GGA GCC GAA CAT CAT AAC CCA TTC GAG AAG        [INTRON 2:    gtaaatttta
Phe Pro Gly Ala Glu His His Asn Pro Phe Glu Lys

                                                                        260
aaactactaa gttctaatgt aattaatttt ttttcag  ]     ATT GCC GAG ACA ATT CGT GAT GCT
                                                Ile Ala Glu Thr Ile Arg Asp Ala

                                        270                             280
CTT GAT GAG TTC TTT CCA GAC ACC AAC AAG CGC CTG TAT CGT GAA CCA GGA AGA TTT TTC
Leu Asp Glu Phe Phe Pro Asp Thr Asn Lys Arg Leu Tyr Arg Glu Pro Gly Arg Phe Phe

                                        290                             300
GCA GCC GGT CCA TTT TCT CTT GTT GCA AAC ATC ATT CAT GCC ACC GAG GTT CCT GCT TCC
Ala Ala Gly Pro Phe Ser Leu Val Ala Asn Ile Ile His Ala Thr Glu Val Pro Ala Ser

                                        310                             320
AAA ATC ACA AAG GAT CCA AAA GAC TGC GCT GAT CAT GGA TAC ATG TAC TAC ATC AAC GAC
Lys Ile Thr Lys Asp Pro Lys Asp Cys Ala Asp His Gly Tyr Met Tyr Tyr Ile Asn Asp

                                        330                             340
GGA GTC TAC GGA TCG TTC AAC TGC ATC CTC TTC GAT CAT GCT CAT CCA ATC GGA TCC CCG
Gly Val Tyr Gly Ser Phe Asn Cys Ile Leu Phe Asp His Ala His Pro Ile Gly Ser Pro

                                        350                             360
CTT TTT GAC ACT GAT CGC AAC GAG AAG TTC ATG TCA ACA ATC TGG GGG CCA ACT TGC GAC
Leu Phe Asp Thr Asp Arg Asn Glu Lys Phe Met Ser Thr Ile Trp Gly Pro Thr Cys Asp

                                        370                             380
AGT CTA GAT CTT GTC GAG GAT AAG AAG TTG ATG CCA AAG ATG AAT GTT GGA GAA TGG CTC
Ser Leu Asp Leu Val Glu Asp Lys Lys Leu Met Pro Lys Met Asn Val Gly Glu Trp Leu

                                        390                             400
TAC TAT CCG GAC ATG GGA GCG TAC ACT CTT GCC GCT GCC ACC ACG TTC AAT GGA TTT TCG
Tyr Tyr Pro Asp Met Gly Ala Tyr Thr Leu Ala Ala Ala Thr Thr Phe Asn Gly Phe Ser

                                        410                             420
AAG CCT GTG CCG ATG TAT GTG ATG AGC GAG GAA ATG TGG GAG AGC ATT CGT GAC TCA ACT
Lys Pro Val Pro Met Tyr Val Met Ser Glu Glu Met Trp Glu Ser Ile Arg Asp Ser Thr


CAT GTT TGA ttc ttg tct aaa tta ttg tgt cgg aaa ata aat att ctt ttg tta
His Val ***
```

Figure 2.1B Sequence of the Tc1 remnant in *pc13*.

```
100                                 108
GTT ATG GGG ACC GGT GTT TCT GCG GAA Cat tgt gtt ttt aga ttt
VAL MET GLY THR GLY VAL SER ALA GLU His cys val phe arg phe

tgt gaa cac tgt ggt gaa gtt tca aaa caa aat aac cac tta gaa
cys glu his cys gly glu val ser lys gln asn asn his leu glu

aaa agt tac aca caa aaa acc aaa agt gga tat ctt ttt ggc cag
lys ser tyr thr gln lys thr lys ser gly tyr leu phe gly gln

120                                                 110
cac tgT ATC ATC TAT GCC AAC CCA TGC AAA ACA CGC AGT
his cys ILE ILE TYR ALA ASN PRO CYS LYS THR ARG SER
```

Figure 2.1C Comparison of ODC sequences of different species.

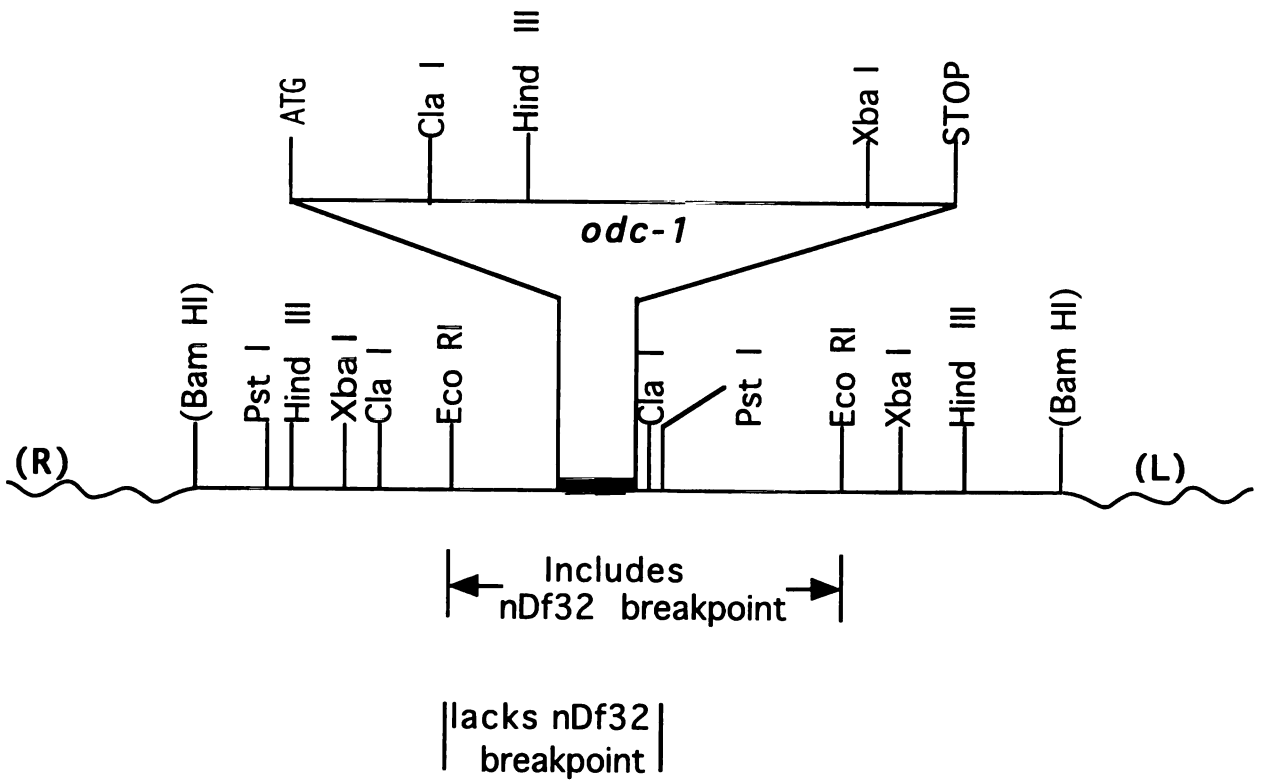
```
                                  100                 110
                                   *                   *
C. eleg (pc13)   GFDCASK DEIDIVMGTGV--SA EH+37+IIYANPCK
C. eleg  (wt)    GFDCASK DEIDIVMGTGV--SA ER----IIYANPCK
Mouse            GFDCASK TEIQLVQGLGV--PA ER----VIYANPCK
Frog             GFDCASK TEIQLVQSIGV--SP ER----IIYANPCK
Chicken          GFDCASK TEIQLVQSIGV--PP ER----IIYANPCK
Trypanosome      GFDCASK TEIQRVRGIGV--PP EK----IIYANPCK
Drosophila       GFDCASK NEVKLVLGFDV--SP ER----IIFANPCR
Yeast            NFDCASK VEIDRVLSMNI--SP DR----IVYANPCK
Leishmania       GFDCASK EEIHMVLGRQLVASP DD----IIFANPCK
Neurospora       GFDCASK AEIEQVLRMGV--DP SR----IIYAQPCK
```

Mapping of a lambda genomic clone harboring *odc-1*

We analyzed the structure of the lambda genomic clones by restriction mapping and by Southern blotting, using probes derived from both 5' and 3' regions of the *odc-1* cDNA open reading frame. Genomic clone 2, consisting of a lambda 2001 vector containing *odc-1* , has a 16-18 kb insert oriented such that the lambda right arm is proximal to the initiation codon of ODC. There is approximately 9 kb of 5' non-coding region and 10 kb of 3' non-coding region (Figure 2.2). On the other hand, lambda clone 1 has a shorter (approximately 1 kb) 5' non-coding region.

Figure 2.2. Restriction map of *odc-1* clone 2 in the lambda 2001 vector. R and L designate the right and left arms of the vector. Positions of the EcoRI fragment that includes and of the EcoRI-Pst I fragment that excludes the *nDf32* breakpoint are shown. Restriction sites were determined by single and double digests and those within the central 8-kb EcoRI fragment confirmed by sequencing.

Figure 2.2


ATG
Cla I
Hind III
Xba I
STOP
odc-1
(Bam HI)
Pst I
Hind III
Xba I
Cla I
Eco RI
Cla I
Pst I
Eco RI
Xba I
Hind III
(Bam HI)
(R)
(L)
Includes
nDf32 breakpoint
lacks nDf32
breakpoint
2 Kb

To determine whether lambda clones 1 and 2 have identical coding sequence and therefore likely arose as distinct copies of the same gene, we PCR amplified clone 1 between ClaI (amino acid position 95) and XbaI (amino acid position 362) restriction sites using primers based on the sequence of clone 2. The sequence of the amplified fragment of clone 1 was identical to the corresponding region of clone 2.

Tc1 insertional mutagenesis of *odc-1*

An *odc-1::Tc1* insertion mutant (*pk32*, described in Zwaal *et al.* 1993) had a Tc1 element inserted in the first exon after the arg 109 codon (Figure 2.1a). As has been described for other Tc1 insertions, a high frequency of somatic excision made this *odc-1::Tc1 (pk32)* allele difficult to work with. Further screening of *pk32* progeny by PCR yielded a spontaneous incomplete excision of Tc1 element and was stable. The structure and phenotype of *pc13* is more fully described below. *pc13* was outcrossed 10 times to the Bristol strain *dpy-11 unc-42* and was then used to map *odc-1*.

Chromosomal localization of *C. elegans odc-1* gene

A *C. elegans* Yac polygrid mapping blot (Coulson *et al.* 1991) was probed with sequences encompassing the full length *odc-1* coding region and revealed two positive Yacs, Y37G2 and Y44A7 (data not shown), indicating that *odc-1* is located in the middle of LG V near the gene *dpy-11*. Fingerprint

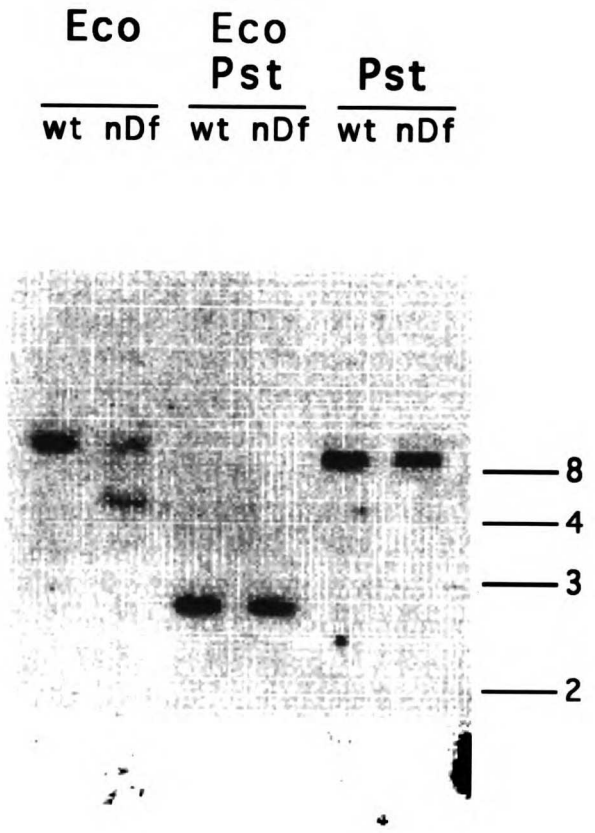
analysis of lambda clones 1 and 2 (A. Coulson, personal communication) was consistent with this conclusion. A three point cross using *dpy-11* and *unc-42* markers was used to determine the genetic map position of *odc-1*. Briefly, the progeny of *+odc-1(pc13) + /dpy-11 + unc-42* heterozygotes were screened for either dpy non Unc or unc non Dpy recombinants. The recombinants were analyzed for the presence of *pc13* by PCR. Among dpy non Unc, 3/17 were *pc13* and among unc non Dpy 10/14 were *pc13*. These data confirm that *odc-1* is between *dpy-11* and *unc-42* on LGV, and is closer to *dpy-11* than to *unc-42*.

The deficiencies *nDf32* and *sDF20* include *dpy-11* (Johnson and Baillie 1991). To reveal the relationship of *odc-1* to the region on chromosome V deleted in *nDf32*, we compared the genomic DNA of worms heterozygous for *nDf32* or *sDf20* with that of a wild-type strain. DNA was cleaved with a variety of restriction enzymes and analyzed by Southern blot hybridization. Hybridization was initially carried out with a 5' *odc-1* probe (a 360-nt SstI fragment comprising the initial 180-nt of the *odc-1* open reading frame and 180-nt of contiguous upstream DNA). EcoRI digestion showed an 8 kb band in both *sDf20* (data not shown) and wild type DNA; this same band plus an additional band of approximately 5 kb was seen with DNA from an *nDf32* heterozygote (Figure 2.3). This EcoRI polymorphism can not be the result of the *eT1* reciprocal translocation that is common to both *nDf32* and *sDf20*. Therefore, the polymorphism shows that *odc-1* is present near the *nDf32* deficiency breakpoint. Quantitative examination of gene copy number in this

region, which falls from 2N to 1N in *nDf32* (P. Sengupta, personal communication) confirmed this conclusion. The breakpoint is positioned within the wild type 8 kb EcoRI fragment.

Figure 2.3. Southern blot analysis of DNA from wild-type and *nDf32* strain. DNA was prepared from N2 Bristol wild-type worms (wt) or from worms heterozygous for the *nDf32* deletion (nDf). The DNAs were restricted with EcoRI, with PstI or with both enzymes, as indicated. Southern blot analysis was performed, using a 360- nt probe that spans the translation initiation codon of the ODC open reading frame. DNA fragments used as size markers (2, 3, 4, and 8 kb) migrated at the positions indicated.

Figure 2.3



Further Southern analysis with the same probe showed a single 8 kb PstI fragment in both wild type and deficiency DNA's (Figure 2.3) indicating that this 8kb PstI fragment does not cover the deficiency breakpoint. Because genomic sequencing reveals the presence of but a single PstI site in or near the coding region, located approximately 500 nt 3' of the stop codon, the other PstI site defining the 8 kb fragment must be approximately 6 kb 5' of the *odc-1* translation start site. This implies that the *nDf32* breakpoint cannot be within the coding region of nor positioned 5' to *odc-1*. Moreover, the EcoRI - PstI double digest of *nDf32* DNA shows, like the *Pst* I digest, a single band (in this case of approximately 2.5 kb) which hybridizes to the 5' *odc-1* probe (Figure 2.3). This implies that the *nDf32* breakpoint must be 3' of the PstI site located in the 3' UTR of *odc-1* and within the approximately 3kb fragment defined by that PstI site and the next downstream EcoRI site. Results consistent with this conclusion were obtained with XbaI, HindIII and XbaI - HindIII double digests and by probing the same blots with a full-length coding region probe (data not shown). Therefore, *odc-1* is located in the middle of LG V with the 3' end of its open reading frame within about 3.5 kb of an *nDf32* breakpoint.

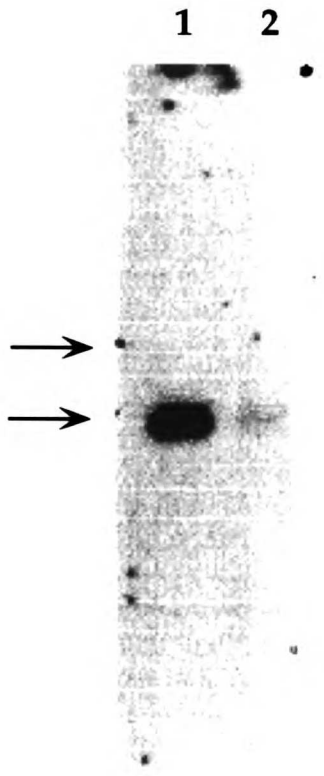
The *odc-1* transcript is trans-spliced

A Northern blot of total RNA or of polyA+ RNA prepared from mixed-stage worms showed a single transcript of about 1500 nt with homology to a full length *odc-1* cDNA coding region probe (Figure 2.4). In *C.*

elegans some but not all mRNAs are formed by a process that includes trans-splicing (Nilsen 1993). The most abundant and first-discovered spliced leader (Krause and Hirsh1987), which is 22 nt in size, is termed splice leader 1 (SL1). We carried out RT-PCR to determine whether the *odc-1* mRNA contained SL1. The primer pair consisted of a 5' (sense) primer corresponding to the initial 20 nt of SL1 and a 3' (antisense) primer initiating at nt 198 within the ODC open reading frame. Two amplification products were obtained, a major band of ~500 nt, and a minor one of ~700 nt. This result, together with the position of the 3' primer within the coding sequence (Figure 2.1a), suggests that the ODC transcript is trans-spliced to SL1 at a position approximately 280 nt [500-(22+198)=280] 5' to the translation initiation codon. To confirm this and to determine with precision the point of splicing, the ~500 nt RT-PCR fragment was cloned and sequenced. The splice acceptor point was found to be 262 nt 5' to the initiation codon, positioned as shown in figure 2.1. The ~700 nt RT-PCR product was not further characterized. RT-PCR was also carried out with an alternative 5' primer, one corresponding to the sequence of a second spliced leader sometimes found in *C. elegans* mRNAs, termed SL2 (Huang and Hirsh 1989; Nilsen 1993; Spieth *et al.* 1993). No RT-PCR product was produced (data not shown).

Figure 2.4. Northern blot analysis. Total RNA was extracted from a worm population composed of a random mixture of larval and adult developmental forms. A portion of the RNA was affinity purified to obtain poly A^+ RNA. The RNAs were subjected to Northern blot analysis using a full-length *odc-1* cDNA probe. Lane 1, 2 micrograms poly A^+ RNA. Lane 2, 10 micrograms total RNA. The arrows mark the positions of 18S and 28S RNA.

Figure 2.4



Expression of ODC activity is under developmental control

To determine whether ODC activity changes in the course of development, eggs were isolated and allowed to mature in synchrony. The cultures were examined periodically to determine their stage of maturity and extracts prepared for analysis of ODC activity. Progressive loss of synchrony is intrinsic in such experiments; we examined sequentially eggs, L1 larva, an L2/L3 pool, an L4/adult pool and, still later, a mixed culture in which synchrony was largely lost. Enzymatic activity was measured by collecting and scintillation counting the $^{14}CO_2$ generated from carboxy-labeled ornithine. Specificity of the reaction was monitored by carrying out parallel determinations with extracts that had been pretreated with DFMO, a mechanism-dependent suicide substrate. Activity was stage-dependent (Figure 2.5). It was higher in L2/L3 than in L1, and higher still in L4/adults, 11.1 nmol/mg protein/hr. The activity was at least 10 times greater in L4/adult stage worms than in eggs or L1 larva.

pc13 is a null allele

To determine the structure of the Tc1 remnant in *pc13*, the gene was amplified by PCR, subcloned and sequenced. Nucleotides 1499 to 1610, the 3'-most end of the Tc1 element (Rosenzweig *et al.* 1983), were found to be inserted within codon 109 of the ODC open reading frame, there replacing the G of CGT Arg triplet (Figure 2.1B). This net insertion of 111 nucleotides,

Figure 2.5. Stage-dependent ODC activity. Eggs were cultured and the resulting synchronized populations sampled periodically, scored by microscopic examination to assess the predominate developmental stage and extracts prepared for determination of ODC enzymatic activity. Activity was determined after preincubation in the absence (cross-hatched bars) or presence (open bars) of DFMO, a specific inhibitor of enzymatic activity.

Figure 2.5

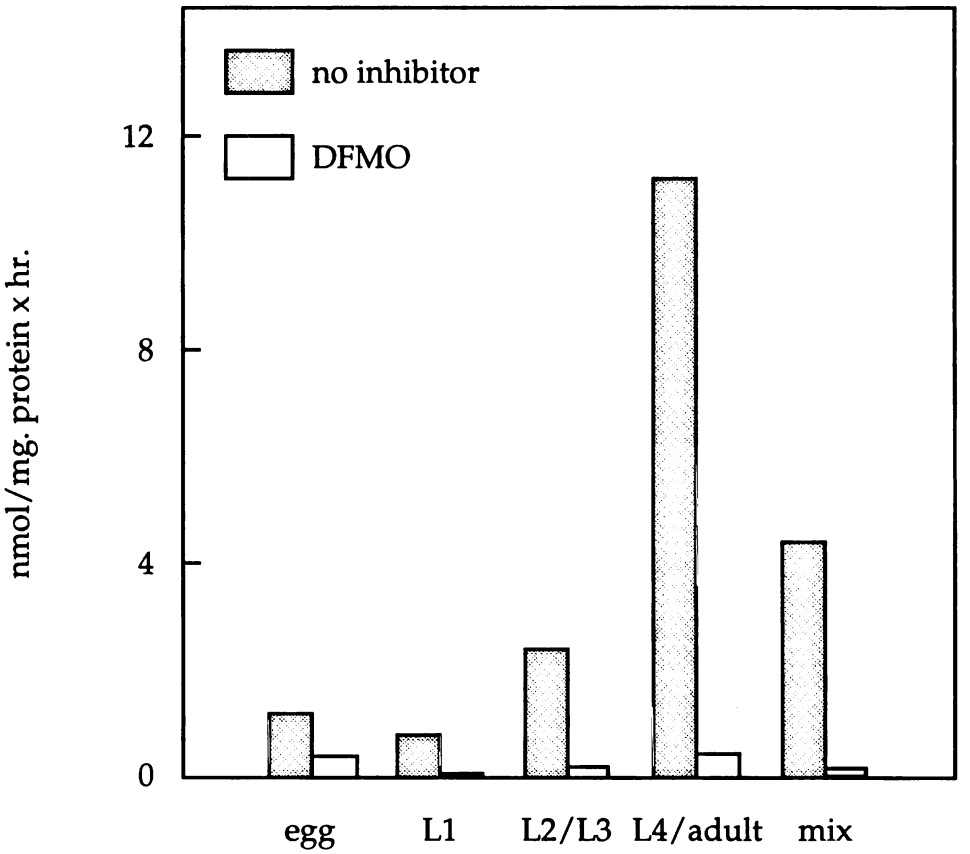

no inhibitor
DFMO
12
8
4
0
nmol/mg. protein x hr.
egg
L1
L2/L3
L4/adult
mix

TABLE 2.1

Experiment	Lysate[a]	cpm[b]	µg[c] protein	cpm-bkgd[d]	cpm-bkgd[e] / protein	mutant / N2 X 100%[f]
1	*pc13*	2,520	70	-124	-1.77	-0.25
	odc-1$^+$	60,111	80	57,356	717.00	
	none	2,644	-			
2	*pc13*	901	270	-533	-1.97	-0.76
	odc-1$^+$	125,273	480	123,836	258.00	
	none	1,434	-			
3	*pc13*	1,769	180	-527	-2.93	-1.12
	odc-1$^+$	61,082	225	58,786	261.30	
	none	2,296	-			
4	*pc13*	2,734	~200	801	4.00	0.79
	odc-1$^+$	103,008	~200	101,079	505.00	
	none	1,929	-			
5	*pc13*	1,171	256	-514	-2.01	-1.30
	odc-1$^+$	30,144	184	28,459	154.67	
	none	1,685	-			

Enzymatic activity of *odc-1(pc13):* N2 (*odc-1*$^+$) and SF1 (*odc-1(pc13)*) extracts were prepared in parallel and enzymatic activity determined.

a: source of extract
b: counts of radioactive product generated by *odc-1*$^+$ or *pc13* extract, or with none present (assay background)
c: micrograms of extract protein used in the assay
d: counts of radioactive product generated minus background
e: column d values divided by column c values
f: column e value for pc13 divided by corresponding value for *odc-1*$^+$, expressed as percent.

although it does not put the ODC coding region out of frame, interrupts the sequence within a highly conserved region (Figure 2.1C) and would be expected to eliminate the enzymatic activity of the encoded mutant protein.

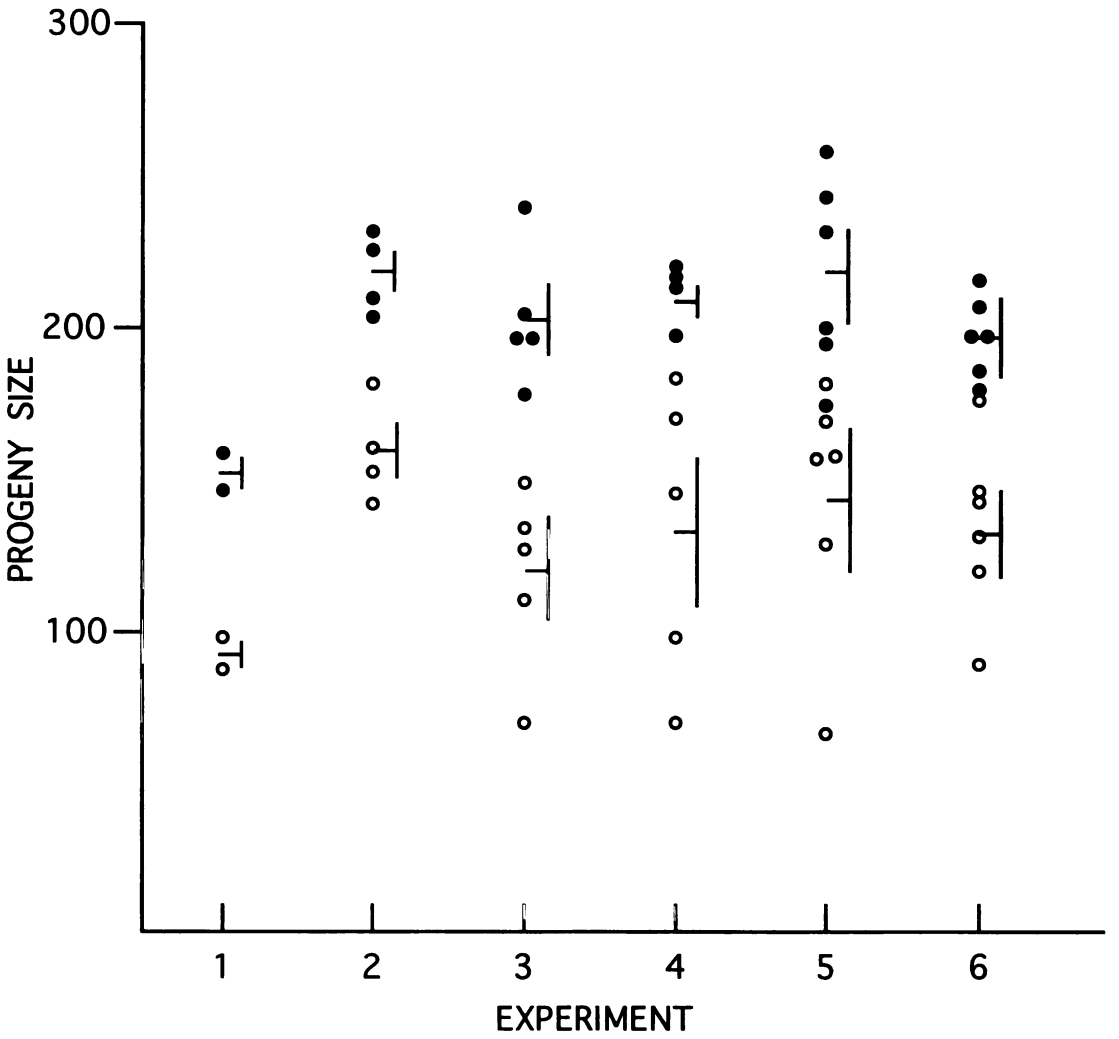
To test this, extracts were prepared in parallel from N2 homozygous *odc-1*$^{+}$ and from strain SF1 homozygous *odc-1 (pc13)* populations consisting predominantly of L4 and adult worms and assayed for enzymatic activity. If mutant extracts truly have no ODC activity, measured values should cluster tightly around the level of assay background (determined with no extract present), sometimes by chance exceeding background and sometimes falling below background. Results were consistent with this expectation (Table 2.1). For the mutants, percent of wild-type activity can be calculated as [(mutant - background)/μg protein]÷ [(wild type - background)/μg protein] X 100%. In five independent experiments measuring mutant and wild-type ODC activity in parallel, mutant activities ranged from -1.30 to + 0.79% of wild-type (n= 5, mean = -0.53%), where negative values represent experiments in which mutant activity registered less than background. We sought in these same experiments to detect authentic ODC activity in mutant extracts by addition of the specific and potent inhibitor DFMO, but found it caused no consistent or significant inhibition (not shown). Activity in control wild-type extracts ranged from about 20- to 100- fold above assay background in different experiments. Assuming conservatively that activity would have been readily detectable in mutant extracts if present at a level equal to or greater than 50%

above background, the ODC activity of the mutants must be reduced at least 40-fold compared with wild-type. More probably, the mutant has no ODC activity.

The *pc13* homozygous worms had no conspicuous phenotype. Neither hermaphrodites nor males were grossly altered in morphology or behavior, and both were fertile. To determine whether ODC deficiency caused a more subtle defect in proliferation, the brood size of mutant hermaphrodites was compared to that of wild-type worms. In six independent experiments, the mutant consistently produced fewer progeny (Figure 2.6); the mean reduction was 35% (range 27-42%, n=6). This suggests that deficiency of enzymatic activity has a weak but real phenotype. We have not, however, ruled out the possibility that the phenotype is due instead to a different gene in the MT3126 mutator strain that remains persistently linked to *pc13* despite outcrossing.

Figure 2.6. Brood size of *pc13* homozygotes and isogenic wild-type worms. Single hermaphrodites were cloned and the total progeny (worms plus eggs) were counted 3 days later. Each symbol represents the brood size of an individual hermaphrodite. Wild-type, closed symbols; mutant, open symbols. Lines indicate group means $\pm$ SD.

Figure 2.6



DISCUSSION

We have cloned and sequenced the gene and cDNA of *C. elegans* ornithine decarboxylase (ODC). The cloned gene encodes a protein of 422 amino acids homologous to ODCs from other species. The production of ODC activity by *in vitro* transcription and translation of the cloned *odc-1* cDNA demonstrates that it corresponds to authentic *odc-1* mRNA. We have shown previously that mouse ODC owes its short intracellular half life to the 37 amino acids located at its carboxy terminus (Ghoda *et al.* 1992). Truncation of this region leaves mouse ODC stable but does not alter its enzymatic activity. The ODC of the African trypanosome *Trypanosoma brucei* lacks this region and is stable in both the native organism and, when expressed by transfection, in animal cells (Ghoda *et al.* 1990). The ODC enzyme described here lacks a carboxy-terminal extension. Based on this, we predict that the enzyme will prove to be stable. However, it is possible that other regions of the molecule could provide a functional destabilizing element, and the conclusion needs to be tested experimentally.

Presence of a single ODC gene has been reported in all species from which the gene has been cloned so far, except Drosophila, which has two (Rom and Kahana 1993). Southern analysis of *C. elegans* genomic DNA shows the presence of a single gene hybridizing to the full length coding region of *odc-1*. This is further confirmed by the Northern analysis which

shows a single mRNA of about 1500 nucleotides hybridizing to *the odc-1* full length probe. The production of eukaryotic mRNAs by trans-splicing was first shown in trypanosomes (van der Ploeg *et al.* 1982). In that organism all the mRNAs share an identical trans-spliced 39-nt 5' sequence. Similarly, many but not all *C. elegans* messages have been shown to be processed in this manner. A variety of 5' trans-spliced donors are used. Among the most common of these is SL1. RT-PCR with a pair of primers, a sense primer homologous to SL1 and an antisense primer homologous to a portion of the *odc-1* open reading frame, produced a product ~500-nt in size. Sequencing this product demonstrated the splice acceptor site to lie 262 nt 5' to the translation initiation codon of ODC. Therefore, some or all of the ODC mRNA is produced by trans-splicing to SL1.

Physical and genetic mapping concurred in placing *odc-1* between *dpy-11* and *unc-42* in the middle of LG V near the *nDf32* breakpoint. Kagawa and colleagues (Sakube *et al.* 1994) have isolated the gene for a ryanodine receptor (*ryr-1)* from *C. elegans.* Sequence analysis of *ryr-1* shows that it is located adjacent to the 5' end of *odc-1* ; there are 1745 nt in the intergenic region between the poly A termination signal of *ryr-1* and the initiation codon of *odc-1.* The two sequences are transcribed in the same direction (H. Kagawa, personal communication). It has been reported that when two genes share close proximity, i.e., with less than several hundred nucleotides intervening, they can produce a polycistronic transcript which is trans-spliced using an

alternate splice leader sequence, SL2 (Spieth *et al.* 1993). We found that RT-PCR using a 5' SL2 primer and a 3' *odc-1* coding sequence primer produce no product. It is likely that the proximity of *ryr-1* and *odc-1* is only a reflection of the worm's small genome size and does not result in production of a polycistronic transcript with ODC as a downstream element.

To investigate the role of polyamines in *C. elegans* development we measured ODC activity in developing worms. Activity rises progressively as larvae advance through development; it is highest in L4 larva/adult worm stages: 11.1 nmoles/mg protein/hour. This may be compared with the activity of ODC found in mammals. Expressed in the same units, adult rat tissues have activities of 0.02 in liver, 1.72 in prostate and 62.3 in androgen-treated male kidney (Pegg and McGill 1979). Worms in late developmental stages thus have activity that lies within the high end of this 3000-fold range. We do not have data on tissue specific expression in *C. elegans.* Changes in global activity could reflect the relative prominence in different developmental stages of cells especially rich in ODC. In mammalian cells, transcriptional as well as post-transcriptional regulation contributes importantly to cell-growth-dependent changes in ODC activity (van Daalen Wetters *et al.* 1989). Quantitative comparisons of mRNA and activity changes during the course of development will indicate whether this is also the case in the nematode.

Perturbing polyamine metabolism will provide a means for assessing their role in worm growth and development. Three means can be used to accomplish this: changing availability of polyamines in the food supply, inhibiting biosynthetic enzymes with drugs such as DFMO and obtaining mutants that alter polyamine metabolism. We have used an *E. coli* strain deficient in polyamine biosynthesis as a nutrient source and find this makes little apparent difference in the growth of wild-type or *odc-1* mutant strains. The complexity of the medium makes it difficult, however, to scrupulously exclude all potential sources of polyamines. Worms grown in medium containing the ODC inhibitor DFMO (0.1 mM) showed no difference in phenotype compared with the wild-type, despite the fact that extracts prepared from worms grown in the presence of the inhibitor had no detectable enzymatic activity (data not shown). Nevertheless, this observation can not assure the effectiveness of inhibition *in vivo*. We have isolated and have begun to characterize a putative *odc-1* null mutant, *pc13*. The genetic restriction of polyamine biosynthesis has resulted in a rather mild phenotype, reduced brood size. We anticipate that restriction of polyamines by dietary limitation will sharpen our ability to educe a stronger phenotype. Indeed, further experiments will be required to establish firmly that brood size reduction results from ODC deficiency rather than a linked mutation in the mutant strain. Rescue experiments with cloned *odc-1* must be done to resolve this question.

It is striking that worms deficient in polyamine biosynthesis are so little perturbed. This result is not inconsistent with previous observations in unicellular organisms or dispersed cultured cells: there, exogenous polyamines fully complement biosynthetic deficiencies. We anticipated, however, a different result in a multicellular organism with an elaborate developmental scheme. That expectation proved mistaken. Surprises of this kind have become commonplace. Phenotypes are commonly mild or different from anticipated in mutants engineered to have knockouts of various conserved genes with functions reasonably presumed important (Shastry 1994). Redundancy of function seems a plausible general explanation of such findings. We have apparently excluded redundancy of ODC itself in *C. elegans*. Another pathway of polyamine biosynthesis, present in plants and in some bacteria, is initiated by enzymatic decarboxylation of arginine. However, we searched for and found no evidence of arginine decarboxylase activity in worm extracts (results not shown). It is therfore unlikely that an alternative means exists in worms for producing the polyamine precursor putrescine. It is likely that the mutant retains means of regulating pools, despite the presumption that any polyamines present must be imported. Mammals have access to multiple mechanisms, including catabolism and transport, for control of polyamine pools (Davis *et al.*1992); these or novel forms of regulation may operate in worms as well. It remains to be fully determined under what conditions ODC activity contributes to fitness.

Chapter 3

Ornithine decarboxylase deficiency in *C. elegans*:

Consequences for embryogenesis

ABSTRACT

Ornithine decarboxylase (ODC) catalyzes the conversion of ornithine to putrescine, an obligate precursor to the polyamines spermidine and spermine. We previously reported that homozygous *odc-1* (allele *pc13*) worms have no detectable ODC activity, yet these mutant worms appear normal, with but a slight reduction in total brood size, when grown in complex medium. We now show that when ODC-deficient worms are transferred to polyamine-free medium, they show a strong phenotype. *odc-1* worms have two different fates depending upon the developmental stage at which polyamines are removed. If the polyamines are removed at the L1 larval stage, the mutant animals develop into adult hermaphrodites that produce very few or no eggs. In contrast, if mutant larvae at the later L4 stage of development are transferred to polyamine-deficient medium, they develop and lay eggs normally. However, approximately 90% of the eggs yield embryos that, although well differentiated, arrest at early stage 3, i.e., morphogenesis, indicative of the 550 cell stage. Either maternal or zygotic expression of ODC provide partial rescue of embryonic lethality. Supplementing deficient medium with the polyamine spermidine prevents expression of all the phenotypic alterations associated with ODC deficiency.

INTRODUCTION

Polyamines are ubiquitous and necessary for cell growth. Although microorganisms or cultured cells from metazoans cannot grow without polyamines, growth resumes when they are provided exogenously (Tabor *et al.* 1981; Macrae and Coffino 1987; Steglich and Schefler 1983). Ornithine decarboxylase (ODC), the enzyme that catalyzes the synthesis of putrescine, a polyamine precursor, is ubiquitous and is highly conserved in structure among eukaryotes (Macrae *et al.* 1995). When ODC activity is perturbed, either genetically or pharmacologically, normal cell growth eventually ceases (Steglich and Schefler 1982; McConlogue and Coffino 1983). This growth inhibition can be overcome in single cell organisms and cultured cells by simply adding polyamines to the culture medium. Thus, cells appear to be indifferent to whether they make or get polyamines, so long as they are available. Is this conclusion, valid for micro-organisms and cultured metazoan cells, also true of multicellular organisms?

Some information relevant to this last question is available. Both biosynthesis and uptake contribute significantly to polyamine pools in rodents (Bardocz *et al.* 1996; Sarhan *et al.* 1989). When pregnant mice are treated with difluoromethyl ornithine (DFMO), a specific suicide inhibitor of ODC, they abort their foeti (Fozard *et al.* 1980). The temporal window of vulnerability to fetal wastage is confined to gestational days 5-8. This suggests

that polyamine synthesis is important for specific developmental events and that uptake from exogenous sources (food, gut flora) may be insufficient. Such an experiment cannot determine whether termination of pregnancy is the result of maternal or zygotic ODC deficiency. *C. elegans* mutant *odc-1::Tc1* (*pc13*) worms, referred to subsequently as *odc-1*, have no detectable ODC activity (Macrae *et al.* 1995). This mutation has only a minor phenotypic effect, a reduction in the number of progeny (Macrae *et al.* 1995). Several mechanisms could in principle account for the relatively benign effect of the mutation: mutant worms utilize a polyamine biosynthetic pathway that does not require ODC, do not have a null *odc* allele, or obtain functionally adequate polyamine stores by uptake. Our previous experiments make the first and second of these mechanisms unlikely (Macrae *et al.* 1995). One way to investigate whether the mutant worms need and can use exogenous polyamines is to investigate the consequences of depriving them of such an external source. We show here that depriving ODC-deficient worms of external polyamines leads to developmental catastrophe.

When *odc-1* worms are transferred to polyamine free medium as L4 larvae, their eggs arrest at early stage 3 of embryogenesis. If polyamines are removed from the medium earlier in development, at the L1 larval stage, development proceeds normally to adult stage, but no embryos are produced. Behaviorly, the adult animal is somewhat sluggish and has the appearance of being starved. Together, these findings suggest that ODC activity is most

critically required during embryogenesis and, furthermore, that exogenous polyamines can override the requirement for ODC activity.

METHODS AND MATERIALS

Preparation of polyamine free bacteria

E. coli HT289 (*spe*A, *spe*B, *spe*C, thr, leu), which is deficient in polyamine biosynthetic enzymes (Tabor *et al.* 1981), was grown overnight in M9 minimal medium (Maniatis *et al.* 1982) supplemented with 100 μg/ml threonine, 410 μg/ml leucine, and 10 μM putrescine. The bacteria were pelleted and washed once with M9 in order to remove excess putrescine. The washed pellet was resuspended in M9 and used to inoculate M9 minimal medium supplemented with threonine, leucine and 8.5 μM cadaverine (1,5-diaminopentane). The cadaverine-supplemented bacterial culture was grown overnight at 37^{0}C.

Media for worm growth

Worms were routinely raised on NG agar plates (also referred to in text as rich medium) seeded with bacteria as described (Wood 1988) with some modifications. *E. coli* HT289 was used as food source. To establish polyamine auxotrophy of *odc-1* strain, worms were transferred to plates containing S-medium (Wood 1988) and 1.7% agarose. These plates, referred to as polyamine deficient from here on, were seeded with HT289 bacteria grown in cadaverine supplemented M9 medium. Worms were incubated at 20^{0}C. To

rescue worms of ODC-deficiency, polyamine- deficient plates were supplemented with either 10 μM putrescine or 10 μM spermidine, or with other concentrations of these compounds where noted. Strain N2 was used as isogenic wild-type control.

Worm strains

Wild-type Bristol strain N2, *dpy-11(e224)*, and *unc-42(e270)* used in this study were obtained from the *Caenorhabditis* genetics center in Minnesota.

Analysis of the sterile adult and the embryonic arrest phenotypes

Single L1 *odc-1* larvae were transferred from NG agar plates to polyamine deficient plates or to identical plates supplemented with spermidine. Larval growth was examined every day. The rate of development of the mutant *odc-1* larvae was compared to that of the wild-type N2 strain. The adult worms were monitored for their ability to produce progeny. The mutant adults were further examined under Nomarski optics to determine the presence of eggs.

To assess the arrest phenotype of mutant embryos, single L4 larvae were transferred from NG plates to individual polyamine-deficient plates supplemented with 0 or 10 μM spermidine. The animals were allowed to lay eggs. Hatching was monitored. Any hatched animals were removed from

the plates. The eggs which did not hatch after 24 hours were scored as arrested. Some of the arrested embryos were examined under Nomarski optics to determine the extent of their development. Wild type N2 strain was used for comparison.

Polyamine measurement

To measure polyamine pools, starved L1 larvae were transferred from rich plates to polyamine deficient plates. The worms were allowed to develop to L4/adult stage and then washed off the plates in M9 buffer and pelleted. Worm pellets were stored frozen at -80^{0}C until used. Polyamine concentration was measured on worm extracts in the laboratory of Dr. Carl Porter (Porter and Bergeron 1983).

Matings

Crosses were carried out by placing an individual L4 male with a single L4 hermaphrodite on seeded NG agar or S-medium agarose plate as needed. Success of mating was scored by the percent of males produced in each cross, which is expected to be 50% for cross-progeny and less than 1% for self-progeny.

RESULTS

Identification of a defined medium lacking polyamines

We have previously described mutant worms (*odc-1 (pc13)*) apparently devoid of ODC activity (Macrae *et al.* 1995). Mutant worms are expected to be auxotrophic for polyamines, but a medium deficient in polyamines is needed in order to test this. *C. elegans* worms are commonly grown in the laboratory on medium supplemented with *E. coli* (Wood 1988). Mutant bacteria that cannot make polyamines are themselves polyamine auxotrophs. We screened a series of polyamine analogs to identify those that sustained growth of mutant *E. coli*, but could not themselves support the growth of mutant worms. Cadaverine (1,5-diaminopentane), an analog of putrescine (1,4-diaminobutane) had this property. *odc-1* worms did not grow on polyamine-deficient medium containing mutant *E. coli* raised on cadaverine, unless further supplemented with putrescine or spermidine (e.g., Table 3.1 and Figure 3.2, below). In contrast, wild type worms grew normally on this medium without further supplementation with polyamines. We refer to defined medium supplemented with *E. coli* grown in this way as "polyamine free", even though strictly speaking it contains an unnatural diamine.

Table 3.1: Spermidine can rescue the sterile adult phenotype of *odc-1* mutant. L1 larvae were transferred from NG to polyamine deficient plates and allowed to grow at 20^{0}C. The number of progeny produced by mutant worms was compared to that produced by wild-type worms. Total progeny produced by each mutant worm supplemented with 0 or 1 μM spermidine was determined. Mutant adults were either sterile or produced very few eggs which arrested as embryos under polyamine deficient conditions; in contrast, mutants supplemented with polyamines produced progeny that were similar in number and viability to the wild-type.

TABLE 3.1

EXPERIMENT		1	2	3	4	5
wt	% gravid[a]	100 (5/5)	100 (8/8)	100 (7/7)	100 (10/10)	100 (5/5)
	Number eggs[b]	172		166		135
odc-1	% gravid	0 (0/10)	0 (0/7)	70 (7/10)	10 (1/10)	40 (2/5)
	Number eggs	0	0	22	0.5	5
odc-1^{spd}	% gravid	100 (7/7)	100 (5/5)	100 (8/8)	N/D	100 (5/5)
	Number eggs	189		192		202

L1 larvae develop into sterile adults in polyamine deficient medium.

a = % gravid adults; in parenthesis: gravid animals / total animals
b = mean number of eggs laid
spd = worms grown on spermidine supplemented plates
N/D = not done

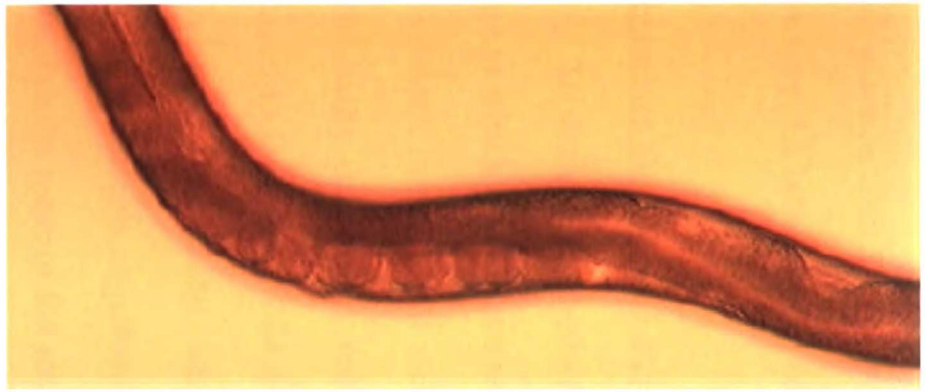
Mutant *odc-1* L1 larvae develop into sterile adults in polyamine deficient medium

To investigate the effect of polyamine deficiency on the development of *odc-1* worms, L1 larvae were cloned on polyamine free plates and their development was observed daily under a dissecting microscope. The rate of development of the mutant worms was compared to that of wild type worms treated identically. The mutant worms completed larval development at about the same rate as wild type. However, in contrast to wild type, the mutant worms contained and laid few eggs or none (Figure 3.1 and Table 3.1). In 3 of 5 experiments a few eggs were apparent in the mutant animals but almost all failed to hatch (Table 3.1). This implies that ODC activity facilitates development to the gravid adult stage, and that enzymatic deficiency results in an incompletely expressed sterile phenotype. Also, the adult mutant worms appeared behaviorly somewhat sluggish. To confirm that the sterile adult mutant phenotype of the *odc-1* strain was indeed due to polyamine deficiency, mutant worms were grown on plates supplemented with either putrescine or spermidine. Animals grown on polyamine supplemented plates produced eggs and progeny like the wild type animals (Table 3.1), demonstrating auxotrophy of the mutant worms and confirming that *C. elegans* requires polyamines for normal development.

To further determine whether the sterility of the mutant worms was due to lack of polyamines, extracts were prepared from both *odc-1* and wild

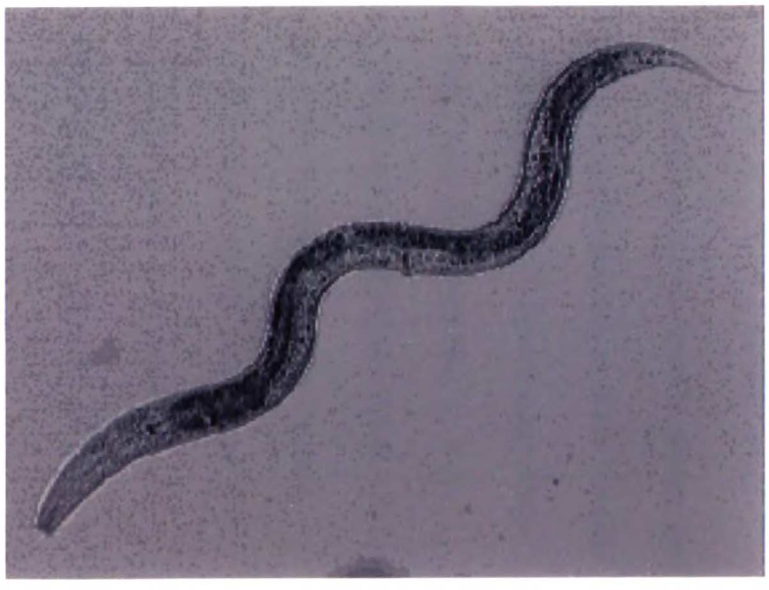
Figure 3.1: Mutant L1 larvae develop into sterile adults. L1 larvae (*odc-1* and wild-type) were transferred from NG to polyamine deficient plates and incubated at 20^{0}C. Growth was monitored. Wild type worms almost invariably grew to adult stage and produced progeny. In contrast, *odc-1* worms arrested as sterile adults. Adult hermaphrodites were viewed under Nomarski optics to confirm the lack of eggs in the gonad of the mutant worm. (a) wild-type adult, (b) *odc-1* adult, and (c) *odc-1* adult under higher magnification.

Figure 3.1

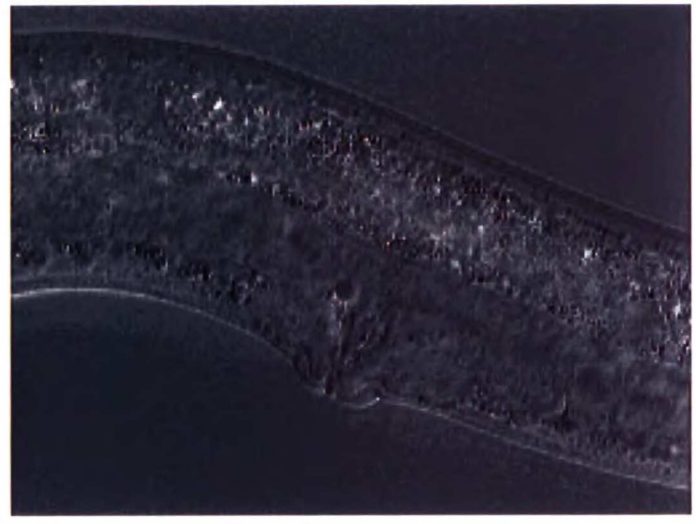


(a) Wild-type Adult

(b) Mutant Adult



(c) Mutant Adult



type worms and polyamines were measured. No putrescine or spermidine were detected in the mutant worms (Table 3.2). In contrast, the wild type worms had levels of putrescine and spermidine more than 200-fold in excess of the minimal levels detectable. The spermidine homolog aminopropyl-cadaverine, but not cadaverine, were present in both mutant and wild type extracts; cadaverine was efficiently metabolized to the homolog, a process that takes place in both *E. coli* and the worms (supporting data not shown). In summary, L1 larvae deprived of polyamines arrest as eggless adults.

Polyamines are required during embryogenesis

To determine whether the arrest phenotype was dependent on the stage at which polyamines were withdrawn, we repeated the same experiment, but using L4 mutant worms in place of L1's. L4 larvae were individually transferred from rich medium to polyamine-deficient plates and allowed to lay eggs. Wild type and polyamine-supplemented mutant worms were studied in parallel. Mutant animals laid significantly fewer eggs than wild type animals or polyamine supplemented mutant animals. Additionally, most of the mutant embryos failed to hatch (Figure 3.2a). A few mutant embryos (4-15%) proceeded further to the pretzel stage and some hatched. The hatched animals developed at the same rate as wild-type but arrested as sterile adults. Therefore, the *odc-1* mutation is completely penetrant but incompletely expressed. Supplementation of *odc-1* worms with

Table 3.2: Determination of polyamine pools in wild type and mutant *odc-1* strains. Starved L1 larvae (wild-type and mutant) were transferred from NG plates to polyamine deficient plates and allowed to develop. Worms were harvested at the L4/adult stage and washed in M9 buffer. Worms were pelleted and stored at -80^{0}C until use. Polyamine pools were measured on worm extracts by HPLC. Pure polyamines were used as standards.

TABLE 3.2

	Polyamine Pools (pmol/μg protein)				
Strain	Putrescine	Cadaverine	Spermidine	Aminopropyl Cadaverine	Spermine
wt	11.55 (+/-1.17)	nd[1]	13.95 (+/-2.35)	16.66 (+/-2.06)	nd
odc-1	nd	nd	nd	26.40 (+/-0.43)	nd

Polyamine pools in worm extracts.

All worm samples were grown with bacteria raised on cadaverin

[1]nd = not detectable at 0.05 pmole/μg protein

polyamines mitigated but did not fully eliminate a catastrophic developmental arrest; approximately 80% of eggs hatched compared to almost 100% for wild-type (Figure 3.2a).

The arrested embryos produced by polyamine-starved hermaphrodites were examined under Nomarski optics (Figure 3.2b). Nerve cells and gut granules were clearly seen. Twitching was observed in the mutant embryos, an indication that muscle cells were formed. Taken together, these data imply that the mutant embryos were generally well differentiated and indicate that embryogenesis proceeded to at least the 550 cell stage in these embryos.

Two steps in *C. elegans* development are specifically sensitive to polyamine depletion

Depending upon when larvae are transferred from complex medium to the polyamine-deficient environment, the mutant worms had two different fates: L1 larvae developed into sterile adults, and L4 larvae laid eggs that usually failed to hatch. Two explanations are possible for these distinct outcomes: (i) the need for polyamines is most restrictive at these two steps or (ii) development stops whenever internal polyamine pools are sufficiently depleted, which happens at a different stage depending on whether external supplies are withdrawn at the L1 or L4 larval stage. To distinguish between these possibilities, we raised worms on different amounts of polyamines

Figure 3.2: Polyamine deficiency results in embryonic arrest. Mutant L4 larvae raised on NG plates were transferred to polyamine deficient plates supplemented with either 0 or 10 μM spermidine. Wild type N2 strain, treated identically, served as control. Worms were allowed to develop and lay eggs. Hatching was monitored. Embryos that did not hatch within 24 hours were scored as arrested. (a) Graphical representation of total number of eggs laid and percent eggs hatched. Each data point represents the progeny of a single worm. (o) number of eggs laid, (•) percent eggs hatched, (-) group mean. (b) Nomarski photomicrograph of wild-type and mutant embryos.

Figure 3.2a

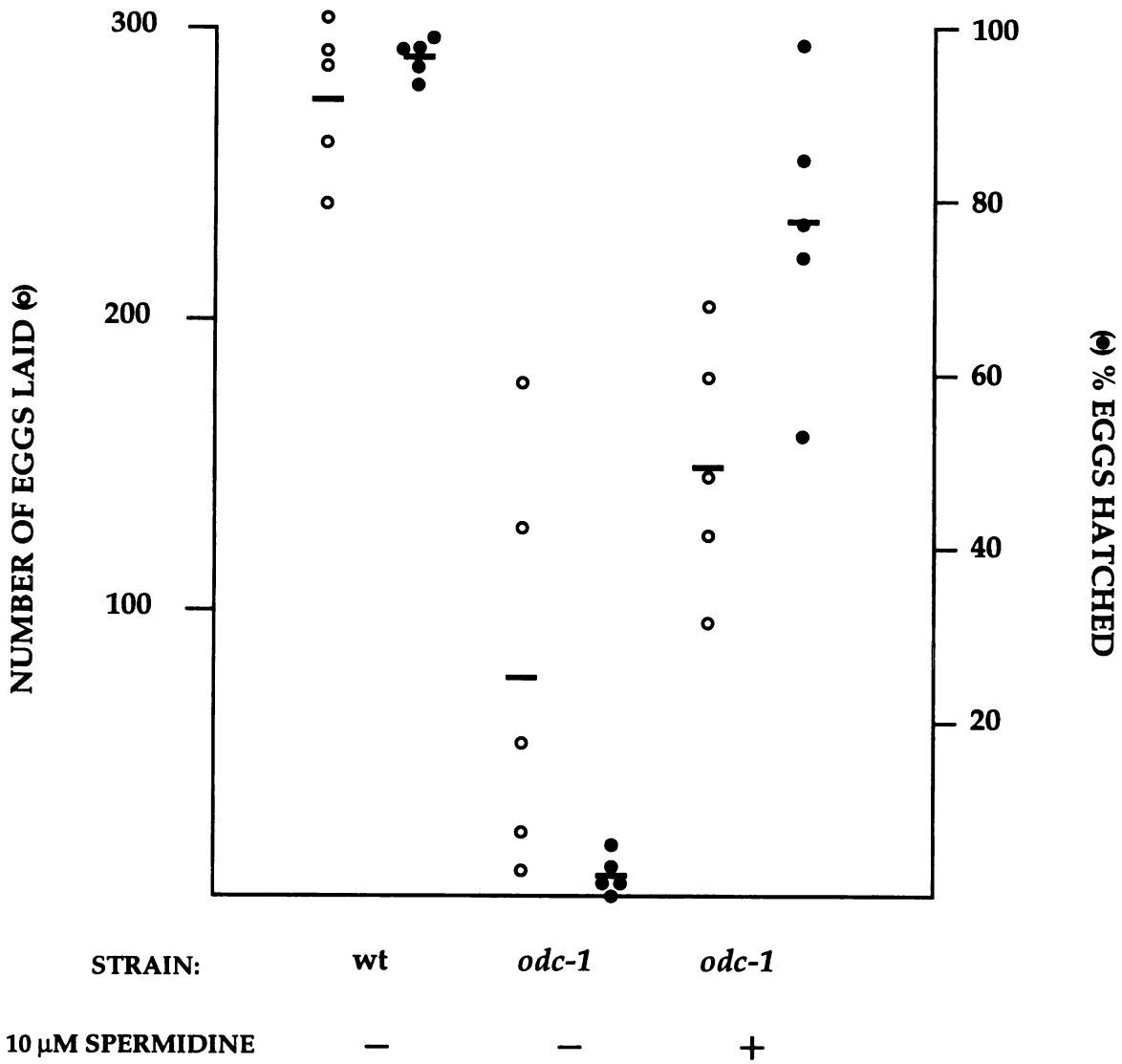
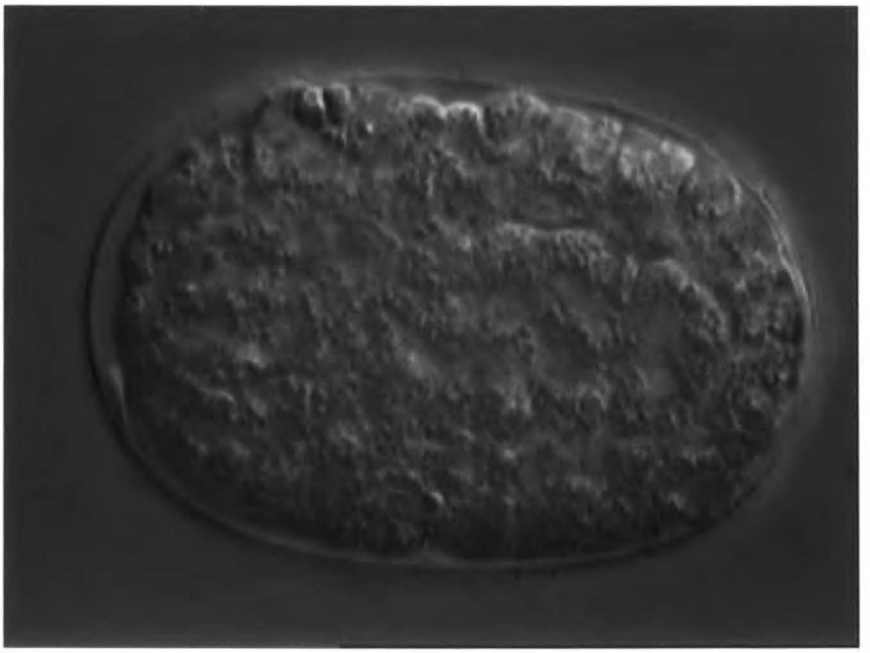
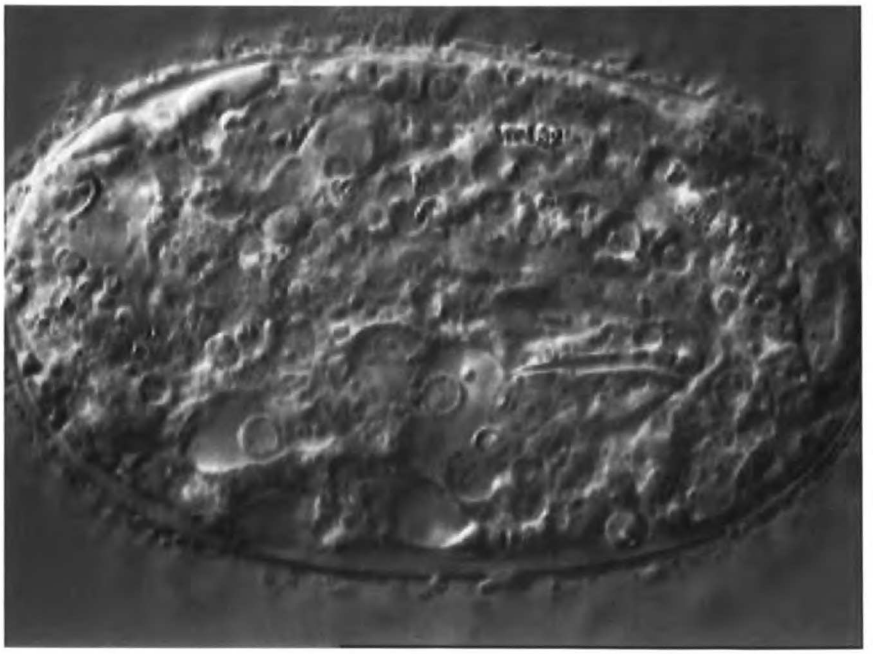

300
200
100
NUMBER OF EGGS LAID (o)
100
80
60
40
20
(•) % EGGS HATCHED
STRAIN:
wt
odc-1
odc-1
10 μM SPERMIDINE
–
–
+

Figure 3.2 b

Wild-type



Mutant



before transfer of L1 larvae to polyamine deficient plates. If hypothesis (ii) is correct, we would expect different degrees of loading of polyamine pools to allow development to proceed to different points.

First, in order to determine the minimum polyamine requirement for proliferation, worms were raised on polyamine-deficient medium supplemented with 0.001, 0.01, 0.1 or 1 μM spermidine. Supplementation with 0.1 μM spermidine was the minimum polyamine concentration sufficient to maintain growth of mutant worms for multiple generations (data not shown). We next pre-loaded worms with polyamines, using supplementation with 0.1, 1 or 10 μM spermidine, and then transferred L1 larvae to polyamine-deficient medium. An average of 9, 55 and 89 eggs were produced by mothers raised on 0.1, 1 and 10 μM spermidine, respectively. We showed above that L1 larvae raised on complex polyamine-rich medium produce sterile adults (except for a few escapers) when transferred to polyamine deficient plates (Figure 3.1 and Table 3.1). In apparent contrast, a reduction in egg production and embryonic arrest is observed here. This difference in outcome will be discussed below. We measured polyamine pools in extracts of worms raised on different concentrations of spermidine to determine whether we had indeed repleted them in a dose-dependent fashion. Table 3.3 shows that we indeed raised the internal polyamine pools. Mutant worms raised on 1 μM spermidine had 5 fold more polyamines than

the ones raised on 0.1 μM spermidine, and mutants raised on 10 μM spermidine had still higher levels of polyamines. This result is consistent with the variable number of progeny produced by *odc-1* worms depending upon the concentration of polyamine supplementation on which the mutant hermaphrodites were raised prior to being transferred to deficient medium; in each case, however, the arrest occured at the embryonic stage. This confirms that polyamines are specifically required for successful development of the *C. elegans* embryo and that a polyamine deficiency results in stage-specific arrest.

ODC deficiency produces sterile adults (Figure 3.1 and Table 3.1). In order to determine the minimum concentration of polyamines required to allow larvae to become gravid adults, we transferred L1 larvae raised on either polyamine-deficient medium supplemented with 0.1 μM spermidine or on rich complex medium to polyamine deficient plates supplemented with 0, 0.01, 0.03, 0.1, 0.3 or 1 μM spermidine. Worms under each condition grew to adult stage. However, worms transferred to plates supplemented with 0.01 μM or higher concentrations of spermidine became gravid; in contrast, worms transferred to polyamine deficient plates grew to sterile adults independent of whether they were raised on plates supplemented with 0.1 μM spermidine or rich medium. This confirms that adequately repleting polyamine pools allows L1s to progress to gravid adult stage. Furthermore, a concentration of 1

μM spermidine provided full rescue, while lesser concentrations provided graded degrees of rescue. This is consistent with the internal polyamine pools: polyamine levels in extracts from mutant worms raised on medium supplemented with 1 μM spermidine are similar to those from wild-type worms (Table 3.3). In conclusion, a polyamine requirement is most crucial to two steps: making gravid adults and successful completion of embryogenesis.

Homozygous mutant progeny of heterozygous hermaphrodites display incompletely penetrant zygotic lethality

Polyamine withdrawal from mutant worms results in developmental arrest, as described above. To determine whether the requirement for ODC activity is maternal or zygotic, we examined progeny of heterozygous *odc-1* hermaphrodites obtained by crossing *dpy-11* males to *odc-1 unc-42* hermaphrodites. *dpy-11* and *unc-42* flank the ODC gene (Macrae *et al.* 1995); in this experiment *unc-42* serves as a marker linked to *odc-1*. If a maternal source of ODC mRNA, ODC protein or polyamines is sufficient, a homozygous *odc* null mutation will have no effect in the first generation and the *odc-1* heterozygotes will segregate according to Mendelian genetics, yielding no deficiency of unc progeny. Figure 3.3 shows instead that deficiency of ODC causes about a 3-fold reduction in the frequency of *unc* progeny compared to a control *unc* heterozygote not ODC deficient,

suggesting that zygotic expression of ODC may be important to assure development of the worm embryo. However, exogenous polyamines can rescue the *odc-1* phenotype (Figure 3.2a). Therefore, zygotic expression of ODC is not absolutely required for worm development, but its deficiency significantly reduces survival.

Zygotic expression of ODC mitigates embryonic lethality

We next asked the converse question: can zygotic expression of *odc-1* prevent the developmental consequences of maternal polyamine deficiency? To answer this question, mating between homozygous wild-type males and homozygous *odc-1* hermaphrodites was compared to homozygous *odc-1* males and homozygous *odc-1* hermaphrodites. Wild-type males were mated to wild-type hermaphrodites for control. For each mating, a single hermaphrodite and one or more males were transferred to polyamine deficient medium as L4 larvae. If the *odc-1* phenotype could be rescued zygotically, cross progeny would be observed among the F1 progeny. Because self fertilization mainly produces hermaphrodites, the success of matings, and therefore of zygotic rescue, could be assesed by scoring the percentage of males produced in the F1 generation. Wild-type males mated to *odc-1* hermaphrodites produced a percentage of males in the F1 generation similar to that in the wild-type X wild-type cross, 30% in both. In contrast, *odc-1* male X *odc-1* hermaphrodite cross produced approximately 7% males.

Figure 3.3: Homozygous progeny of heterozygous mutant mothers fail to complete development in polyamine deficient medium. Seven ODC wild-type homozygous *(+ + unc-42/dpy-11 + +)* or ODC mutant heterozygous*(+ odc-1 unc-42/dpy-11 + +)* L4 larvae were transferred to polyamine deficient plates (one/plate) and allowed to lay progeny. *dpy-11* and *unc-42* served as closely linked genetic markers. Three days later, the percent of homozygous dpy (o) and unc (•) progeny produced by both strains was determined. The mean number of progeny produced by ODC wild-type homozygous and ODC mutant heterozygous mothers were 166 (range 100-235) and 180 (range 140-201), respectively.

Figure 3.3

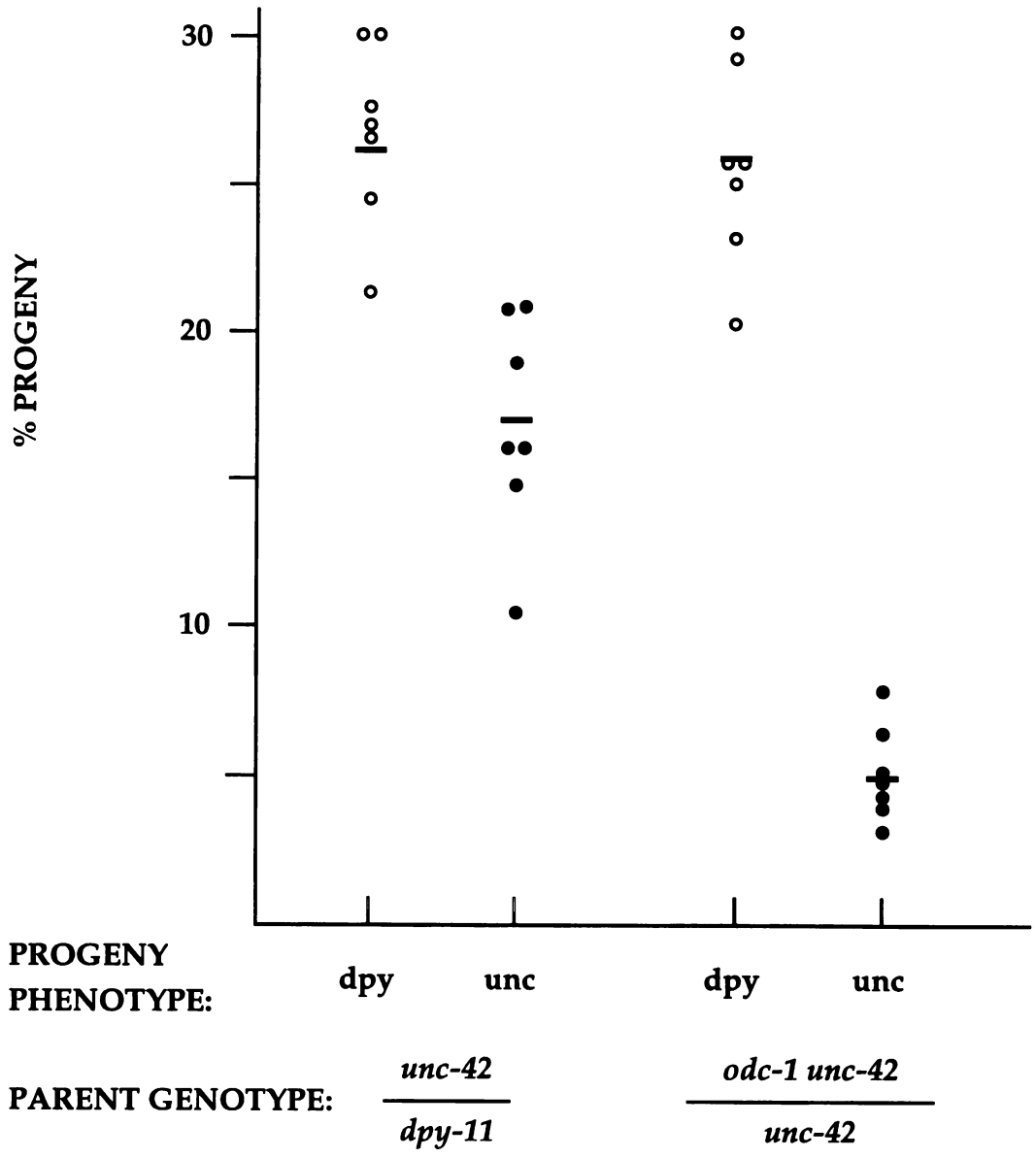
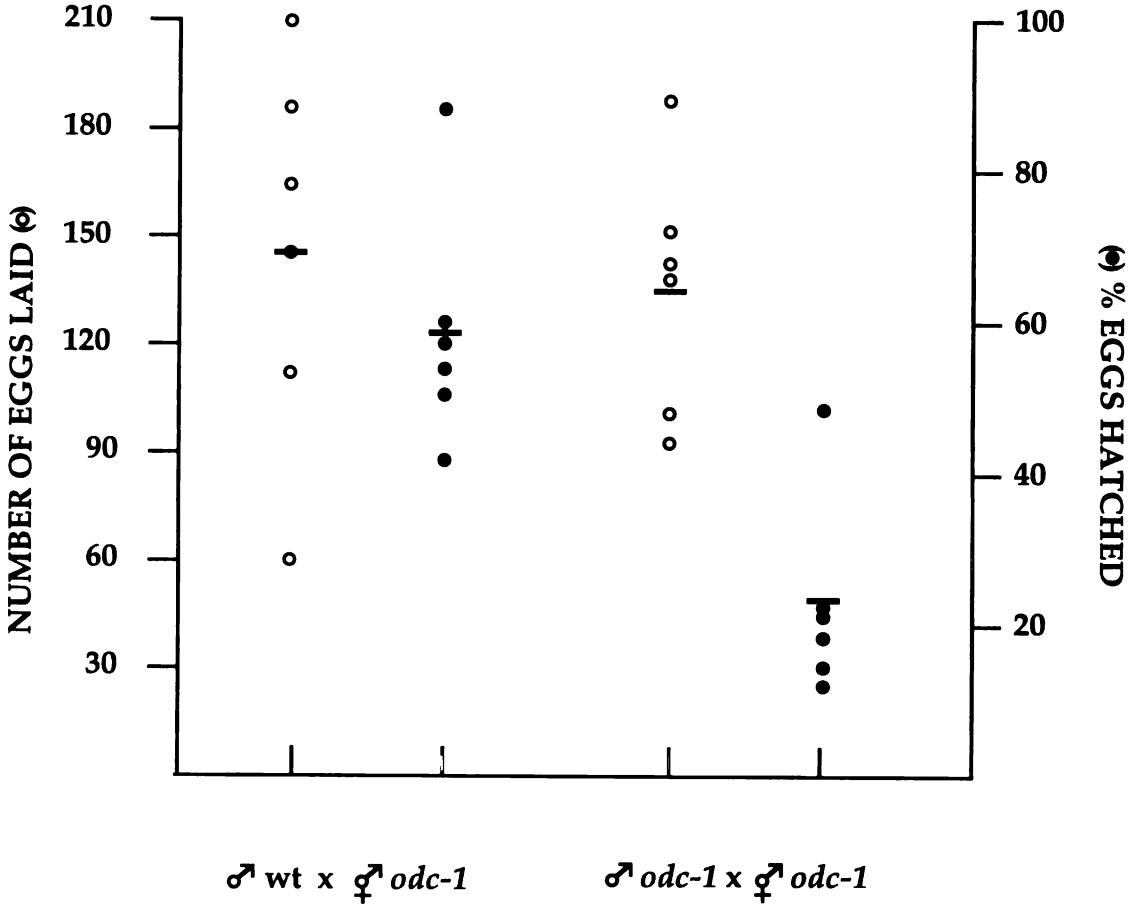

30
20
10
% PROGENY
PROGENY PHENOTYPE:
dpy
unc
dpy
unc
PARENT GENOTYPE:
unc-42
dpy-11
odc-1 unc-42
unc-42

Figure 3.4: Wild-type males can partially rescue *odc-1* embryonic arrest phenotype. Mutant hermaphrodites were crossed to either wild-type or mutant males. Matings were performed on polyamine deficient plates with 1 hermaphrodite and 1-4 males/plate. The number of eggs laid per hermaphrodite (o) and the percent eggs hatched (•) was determined.

Figure 3.4


210
180
150
120
90
60
30
NUMBER OF EGGS LAID (o)
100
80
60
40
20
(•) % EGGS HATCHED
♂ wt x ♀ odc-1
♂ odc-1 x ♀ odc-1

Furthermore, the viable progeny produced by the cross wild-type male X *odc-1* hermaphrodite was significantly higher than that produced by the cross *odc-1* male X *odc-1* hermaphrodite (Figure 3.4). This suggests that wild type males can indeed provide rescue from embryonic lethality. However, the rescue was incomplete (approximately 59% of the eggs hatched as comapared to almost 100% for wild-type X wild-type cross). The number of eggs laid in the wild-type male X *odc-1* hermaphrodite cross was similar to that of *odc-1* X *odc-1* cross (an average of 147 and 134, respectively), suggesting that ODC expression is not needed to start embryogenesis but is needed to successfully finish this process.

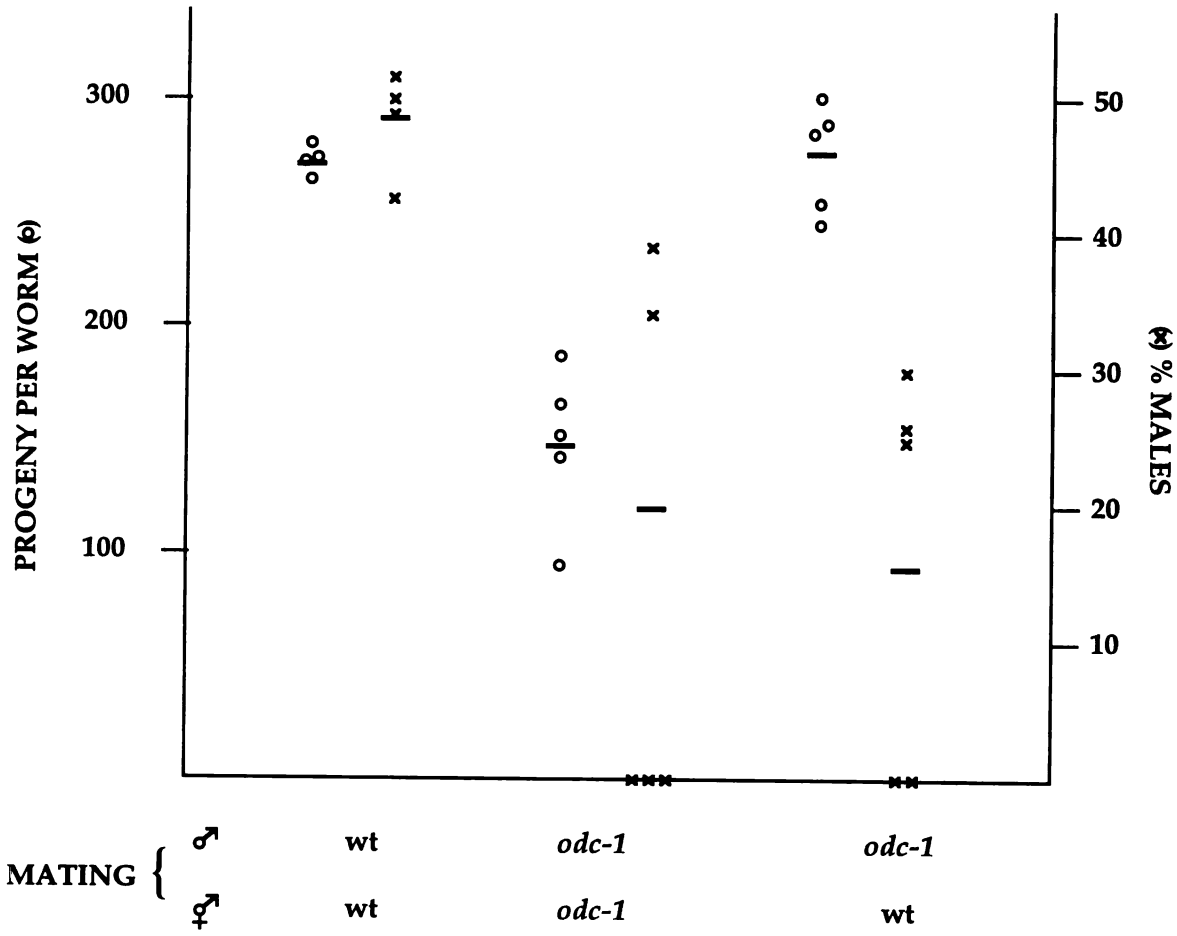
ODC deficiency impedes male fertility

To determine whether ODC deficiency affects male fertility as well as that of hermaphrodites, we mated *odc-1* homozygous hermaphrodites with *odc-1* homozygous males. Matings were performed on polyamine-rich medium (NG plates). As above, the number and fraction of male progeny was used to assess the efficiency of cross-fertilization. A control consisting of a wild-type to wild-type cross yielded the expected number of progeny (almost 300) and fraction of males (43 to 52%) among five independent matings. However, the male fraction was lower, and quite variable, when ODC deficient males were mated to either *odc-1* or wild-type hermaphrodites

(Figure 3.5). This suggests that ODC activity may be important for fertility in males, even if, as here, the medium is replete with polyamines. The difference in number of progeny between *odc-1* male X *odc-1* hermahrodite and *odc-1* male X wild-type hermaphrodite crosses is a reflection of lower number of self progeny produced by*odc-1* hermaphrodites in rich medium (Macrae *et al.* 1995 and chapter 1).

Figure 3.5: *odc-1* males do not mate efficiently on rich medium. *odc-1* males were mated to either mutant or wild-type hermaphrodites. Mating of wild-type males X wild-type hermaphrodites was performed in parallel as a control. Crosses were performed by mating one male with one hermaphrodite per rich medium plate. Success of mating was determined by the percent of males produced in the F1 generation.

Figure 3.5


300
200
100
PROGENY PER WORM (●)
50
40
30
20
10
(×) % MALES
MATING
♂
♀
wt
wt
odc-1
odc-1
odc-1
wt

DISCUSSION

Polyamines are ubiquitous in living organisms. Eukaryotic ODC activity is elaborately regulated in species ranging from yeast to humans, suggesting the utility of precise adjustment of polyamine pools. It was therefore surprising that *C. elegans odc-1* mutants grew normally and showed no adverse effects, except for a slight reduction in progeny number. Since *odc-1* worms have no detectable ODC activity we postulated that *odc-1* worms contain polyamines provided by uptake from the environment. To determine whether this was the case and to further examine the effect of pool depletion, we sought to establish polyamine-free conditions for the growth of worms.

C. elegans is usually raised in the laboratory on a medium supplemented with *E. coli* (although *C. elegans* can be grown axenically, the worms grow very slowly). In order to obtain bacteria devoid of naturally occurring polyamines, we used a mutant strain that cannot synthesize them. These bacteria, however, grow poorly unless their medium is supplemented with polyamines (Macrae and Coffino 1987). We tested a number of polyamine analogs in order to find one that would support growth of the bacteria but not the worms. Moreover, this analog had to be free of any toxic effects on worm growth. Cadaverine, (1,5-diaminopentane), with an alkyl chain one carbon longer than putrescine (1,4-diaminobutane), supported the

growth of *E. coli*, and neither supported nor adversely affected worm growth. Using the medium as described, *odc-1* worms were found to be strictly auxotrophic for polyamines; depletion results in stage-specific growth arrest. This observation confirms our previous conclusions, based on genetic and biochemical evidence, that *odc-1 (pc13)* is a null mutation and that ODC activity is a prerequisite for polyamine biosynthesis in *C. elegans*.

We found that polyamine depletion resulted in one of two fates, depending upon when it was imposed. Earlier depletion, at the L1 stage, resulted in animals that were morphologically adult but failed to produce eggs. These mutant animals looked starved and somewhat sluggish. The failure to produce eggs was consistent with our previous data (Macrae *et al.* 1995, chapter 2) which showed that ODC levels are highest at L4/adult stage. In contrast, mutant worms transferred to polyamine deficient medium at the L4 stage produced embryos that failed to hatch. The embryos were apparently well differentiated but arrested at about the 550 cell stage. Some embryos continued development beyond this stage: in some experiments as many as 20% hatched and developed to the adult stage at approximately the same rate as the wild type animals, except that the mutant animals never became gravid. This implies either that mutant embryos are heterogeneous in their polyamine stores, or that development can proceed unimpeded if embryos stochastically escape stage-specific arrest. Escapers traverse subsequent developmental landmarks with impunity, until they arrive at the next of the

two arrest points identified here. This observation further supports the conclusion that polyamine depletion causes stage-specific defects.

ODC activity may be required to proceed efficiently beyond the 550 cell stage, or its deficiency earlier in embryogenesis may cause a phenotypic effect that only becomes apparent later. The *par* mutation exhibits such a phenotype: PAR activity is required very early during embryogenesis to determine the cell cleavage pattern (Kemphues *et al.* 1988), but *par* embryos arrest late and are differentiated. To distinguish whether the requirement for expression of ODC activity was maternal or zygotic, we mated homozygous *odc-1* hermaphrodites with wild-type males in polyamine-deficient medium. Wild-type males incompletely rescued the *odc-1* phenotype. This implies that *odc-1* does not produce a strict maternal effect and that zygotic expression of ODC is necessary for worm development. Conversely, *odc-1* heterozygotes produced homozygous mutant progeny, but with about a three-fold reduction in number. Furthermore, exogenous polyamines can rescue the *odc-1* phenotype (Figure 3.2). Taken together these results indicate that although zygotic expression of ODC is required for worm survival, it is not always sufficient. Apparently, either maternal or zygotic expression of ODC or polyamine supplementation can augment the yield of viable progeny.

While all our current experiments consistently show that the embryonic arrest occurs in the F1 generation, initially, in some experiments, the arrest phenotype occured in the F2 generation (data not shown). We do

not understand the reason for this difference. We can only speculate that polyamine depletion in our earlier experiments was less complete. Indeed, a comparison of Tables 3.2 and 3.3 suggests that effectiveness of polyamine exclusion varies among experiments.

A polyamine requirement for embryogenesis was not a total surprise. Fozard, *et al.* (1980a) found that ODC activity and polyamine levels spike transiently in the mouse gastrula. Furthermore, when mice were treated with the ODC inhibitor DFMO during days 5-8 of pregnancy, the embryos were reabsorbed (Fozard *et al.* 1980a). The contragestational effects of DFMO were further confirmed in rat and rabbit (Fozard *et al.* 1980b). Lowkvist *et al.* (1980) noticed two peaks in the activity of the ODC enzyme and polyamine levels in chicken embryos: one just before gastrulation and the other before early neurulation. Similar to the results of Fozard *et al.*, inhibition of ODC activity and polyamine biosynthesis in early chicken embryos blocked their development at gastrulation (Lowkvist *et al.* 1980). In our previous experiments, we did not detect high levels of ODC activity in embryos, perhaps because we measured the ODC activity on pooled unstaged embryos.

L4 mutant larvae that were deprived of polyamines became seemingly normal adults, but produced inviable embryos. Our result raises an important question: what is the mechanism of developmental arrest in the *odc-1* mutant embryos? They may have a defect in cell migration, adhesion or transformation of cell fate. Lineage experiments could help determine

which of these possibilities contribute to the arrest phenotype of the mutant embryos under polyamine deficient conditions.

Polyamine deprivation is deleterious to worms. Different means and degrees of reducing polyamine pools yield effects of graded severity. In order of decreasing severity of phenotype: [1] *odc-1* mutation leads to developmental arrest in polyamine free medium. The arrest point depends on when polyamines are removed (Figures 3.1 and 3.2). [2] Either maternal or zygotic expression mitigates the effect of deprivation, but inefficiently. Maternal expression alone (Figure 3.3) reduces yield of viable progeny about three-fold compared to normal expression. Paternal expression alone reduces the yield of viable progeny to about two-fold compared to normal expression (Figure 3.4). [3] In the absence of any ODC activity, polyamine supplementation (using rich medium, Macrae *et al.* 1995 and chapter 1) or spermidine supplementation of minimal medium (Figure 3.2a) reduces the yield of progeny by 20-30%. [4] Heterozygosity for an *odc-1* mutation has no apparent effect; *odc-1* is haplo-sufficient in the worm.

Complete restoration of the wild-type phenotype was never observed. This could be due to failure to deliver polyamines at optimal times and amounts. Although, worms can transport polyamines from the medium, it appears that transport may not be as effective as biosynthesis of polyamines (Table 3.3). Alternatively, there may be another mutation tightly linked to

Table 3.3: Preloading worms with varying concentrations of spermidine repletes polyamine pools in a dose-dependent fashion. Mutant worms were raised on polyamine deficient plates supplemented with 0. 0,1, 1 and 10 micro molar spermidine until L4/adult stage. Wild-type worms without supplementation were analyzed in parallel. Worms were harvested and polyamines determined as before.

TABLE 3.3

Worm Treatment with spermidine	Polyamines pools (pmoles/μg protein)			
	putrescine	cadaverine	spermidine	Amino-propyl cadaverine
WT + 0 μM	39	<2	53	49
mutant+ 0 μM	<2	14	4	63
mutant+ 0.1 μM	<2	12	5	49
mutant+ 1 μM	3	7	23	52
mutant+ 10 μM	17	<2	27	45

Polyamine pools in worm extracts raised on varying concentrations of spermidine.
All worm samples were grown with bacteria raised on cadaverine.

odc-1, despite the fact that the *odc-1::Tc1* strain used to generate *odc-1 (pc13)* was out-crossed more than ten times (Macrae *et al.* 1995). Rescue experiments with injection of the cloned ODC gene could differentiate between the two possibilities. Also, polyamines can not directly rescue embryos (data not shown), but permeability barriers may make such results meaningless.

L1 larvae raised on 0.1, 1 and 10 μM spermidine before transfer to polyamine deficient plates produced an average of 9, 55 and 89 eggs, respectively, which uniformly failed to hatch. In contrast, L1 larvae transferred from complex medium to polyamine deficient plates routinely developed into sterile adults except for a few escapers (Table 3.1). There are at least four possibilities that could contribute to this difference, (1) the effective concentration of polyamines in complex medium is less than that provided on spermidine supplemented plates, (2) polyamine pool measurement in worm extracts is not a true reflection of available polyamines in the relevant compartment, (3) the number of eggs produced by worms raised on low concentrations of polyamines (i.e. circa 0.1 μM) is a graded function of that concentration, (4) polyamine exclusion from the deficient medium is not uniformly effective among experiments. We ruled out the first possibility by measuring the polyamine pools of worms raised on rich medium or on 1μM spermidine supplemented plates. Polyamine pools were found to be similar in these two cases (Putrescine levels were 2.1 pmol/μg of protein for each, and

spermidine levels were 23.2 and 24.6 pmol/μg of protein for complex medium and spermidine supplemented plates, respectively). A combination of the second, third and fourth possibilities therefore appears likely to account for the difference in egg production by L1 larvae transferred from rich versus polyamine-supplemented minimal plates. In support of the fourth possibility, variability in polyamine exclusion from the deficient medium did appear among experiments (Tables 3.2 and 3.3).

When polyamines are removed from the medium at the L1 stage of development, the worm develops into a sterile adult. *spe* mutants also produce a sterile adult phenotype. This mutation is due to defects in sperm production and can be rescued by mating with a wild type male (Ward and Miwa, 1978; Argon and Ward, 1980; Ward *et. al.*, 1981). Since *odc-1* phenotype could not be rescued completely by mating with wild-type males, it suggests that the *odc-1* phenotype is not due solely to defective sperm.

odc-1 males do not mate efficiently on polyamine-rich plates. This phenotype is not completely penetrant; among individual crosses, some mutant males did not mate at all, some mated inefficiently while others mated like wild-type. In contrast, wild type males uniformly mated very efficiently. Polyamines were first described by Leeuwenhoek in the 17th century as crystalline inclusions in human seminal fluid. Transgenic mice that produce ODC in excess are phenotypically normal, but for impaired spermatogenesis (Halmekyto *et al.* 1991). It is striking that worms provided

with access to exogenous polyamines are moderately impaired in hermaphrodite fertility, and more severely defective in male fertility.

CONCLUSION

Polyamines have long been thought to be important for normal cell growth. The requirement for a functional ODC protein along with the complexity of its regulation point towards the importance of ODC and polyamines in cell growth. Knock-out mutations in the *odc* gene of bacteria and yeast have not revealed its significance in cell growth. I have used a genetically well-defined and developmentally more complex organism, *C. elegans*, to study the phenotype of an *odc-1* mutant.

The data shows that *odc-1 (pc13)* is a null allele by three different criteria: (a) the ODC protein produced by the mutant allele is disrupted in a highly conserved region, (b) mutant worms have no detectable ODC activity, and (c) *odc-1* mutation in worms leads to developmental arrest in polyamine deficient medium. One micro molar spermidine can rescue all phenotypes associated with ODC deficiency. In addition, *odc-1* mutant worms appear to grow and reproduce normally on rich medium except for a slight reduction in brood-size. This suggests that worms, like single cell organisms, can transport polyamines from their environment. This was confirmed by preloading worms with increasing concentration of spermidine and measuring the polyamine pools in worm extracts. I found that polyamine levels in worm extracts were repleted in a dose dependent fashion. Therefore, it does not matter if worms cannot make polyamines, so long as they can get them. A

requirement for polyamines is fundamental to development, at least in *C. elegans*, but the capacity for biosynthesis, and its regulation, are redundant.

odc-1 loss of mutation manifests itself at two specific developmental points depending upon when the polyamines are removed from the growth medium: when mutant worms are transferred to polyamine depleted medium as L1 larvae they grow into sterile adults, while L4 larvae grow up to produce eggs which arrest at the 550 cell stage. The mutant worms do not arrest immediately in response to polyamine removal from the medium, but rather the arrest phenotype is specific to two developmental stages. This suggests that polyamines may be specifically required for at least two functions during the worm development: egg production in adults and successful development of the embryo.

The characterization of *odc-1* phenotype will help facilitate the identification of the precise role of polyamines in the worm development. For example, lineage studies could be performed to determine the mechanism of embryonic arrest in the progeny of mutant worms. These experiments would be required to be done in polyamine deficient conditions, which I have established. Expression studies of the ODC mRNA and activity during different stages of the worm development could further help determine when during development ODC activity is required.

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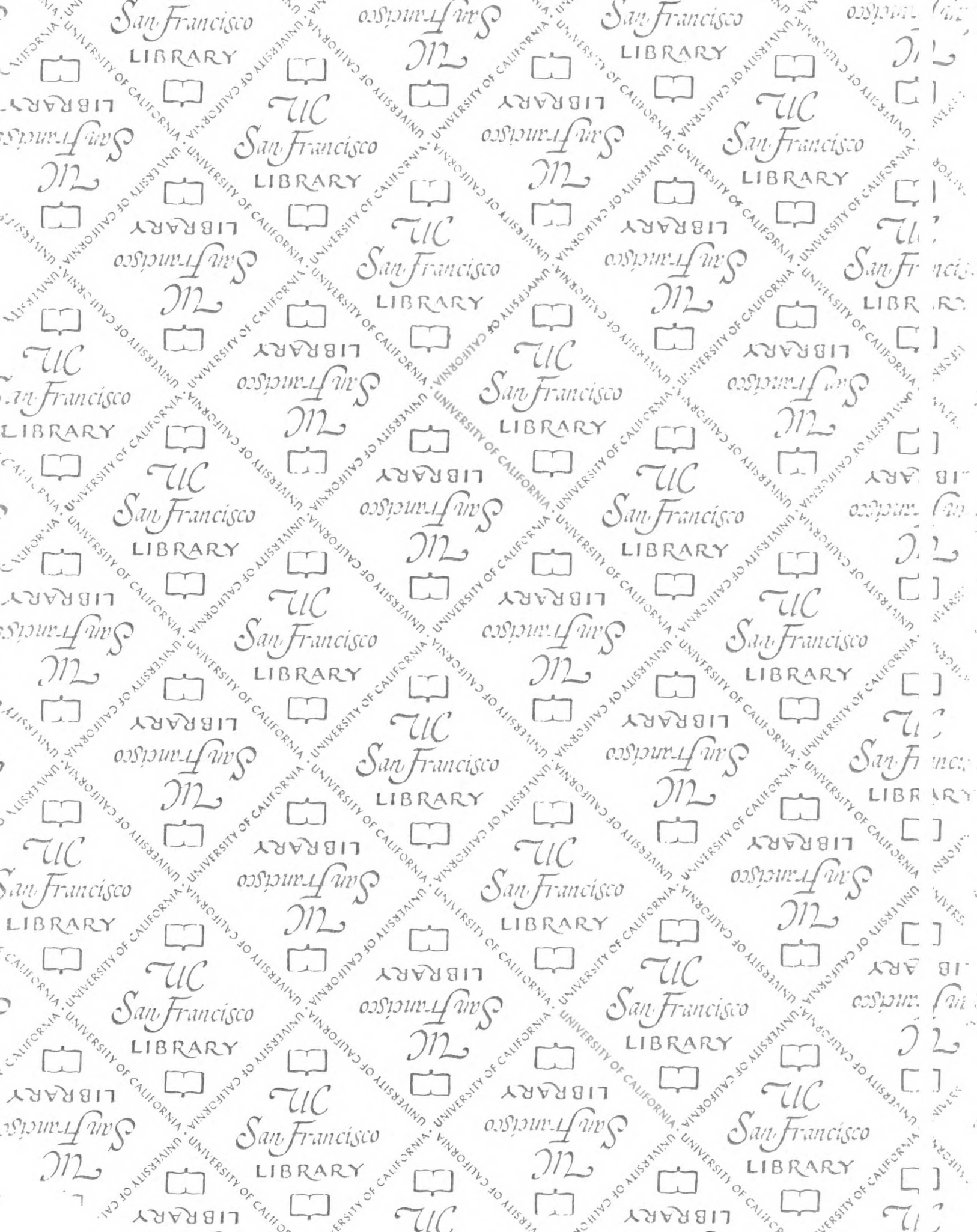
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