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21. The use of cryptic and authentic 3′ splice sites was determined by ribonuclease protection assays (*32*).
22. The cDNAs of *tra* (*13*) and *tra-2* (*14*) were cloned into the baculovirus expression vector pJVP10Z. Recombinant viruses producing Tra or Tra-2, respectively, were generated and purified (*33*). After 72 hours of infection, cells were lysed in buffer A [20 mM tris-Cl (pH 7.5), 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml)] at 4°C for 30 min. The lysate was centrifuged at 8000 rpm (HB-4 rotor) for 30 min. The pellet containing Tra and Tra-2 was extracted in buffer B [10 M urea, 20 mM tris-Cl (pH 7.5), 100 mM KCl, 0.2 mM EDTA, and 1% β-mercaptoethanol] at room temperature for 1 hour. The extract was centrifuged at 50,000 rpm (SW55 rotor) for 1 hour. The supernatant containing Tra and Tra-2 was chromatographed on P11 columns (Whatman, Maidstone, Kent, England) equilibrated with buffer B100 [6 M urea, 20 mM tris-Cl (pH 7.5), 100 mM KCl, 0.2 mM EDTA, and 14.4 mM β-mercaptoethanol]. Tra was eluted with B300 [6 M urea, 20 mM tris-Cl (pH 7.5), 300 mM KCl, 0.2 mM EDTA, and 14.4 mM β-mercaptoethanol]. The flowthrough of P11 containing Tra-2 was chromatographed on DEAE-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with B100. Tra-2 was eluted with B300. The P11 B300 fraction of Tra and the DEAE B300 fraction of Tra-2 were dialyzed against BC850 [20 mM tris-Cl (pH 7.5), 850 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT] at 4°C overnight. Both Tra and Tra-2 were quantified by Coomassie staining on SDS–polyacrylamide electrophoresis gels. As a control, lysate from a recombinant baculovirus expressing the *Drosophila bicoid* protein was subjected to the same fractionation procedures as Tra and Tra-2, respectively. These control protein preparations did not affect splicing.
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34. D1 was transcribed from T7S/R linearized with Dra I. We constructed T7S/R by cloning into the SP73 vector (Promega) a 1775-bp Sty I–Eco RI fragment starting 90 bp upstream from the 5′ splice site of exon 3 and ending 1175 bp downstream from the 3′ splice site of exon 4.
35. The splicing reaction was set up in a total volume of 25 μl with 100 μg of HeLa cell nuclear extract, 12 mM tris-Cl (pH 7.5), 72 mM KCl, 0.12 mM EDTA, 4% glycerol, 3.2 mM $MgCl_2$, 1 mM adenosine 5′-triphosphate, 20 mM creatine phosphate, 3% polyvinyl alcohol, 1 ng of pre-mRNA, Tra, and Tra-2 in the amounts specified elsewhere. The reactions were incubated at 30°C for 2 hours unless otherwise specified, and the RNAs were subsequently analyzed on 6% polyacrylamide gels containing 8 M urea (*24*).
36. D2 and D3 were transcribed from T7S/R linearized with Mlu I and Fsp I, respectively.
37. G(py)/D and G(bps)/D were transcribed, respectively, from SP6G(py)/D and SP6G(bps)/D linearized with Dra 1. SP6G(py)/D and SP6G(bps)/D were constructed by replacing the Sac I–Hinc II fragment in SP6RV/RI with the Hind III–Sfa NI fragments from G(py) and G(bps), respectively. SP6RV/RI was constructed by cloning into the pGEM7Z vector (Promega) a 2075-bp fragment starting 300 bp upstream from exon 3 and ending 1578 bp downstream from the female 3′ splice site.
38. D5, D6, and D7 were transcribed from T7F540 linearized with Eco RI, T7Afl linearized with Eco RI, and T7S/R-M/B linearized with Cla I, respectively. We constructed T7F540 and T7Afl by inserting the 540-bp Fsp I and the 360-bp Afl II fragments from *dsx* exon 4 into the Bluescript SK^+ vector (Stratagene). T7S/R-M/B was constructed by deletion of a 585-bp fragment containing all six copies of the 13-bp repeat sequence from the T7S/R.
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Body-Wall Muscle Formation in *Caenorhabditis elegans* Embryos That Lack the MyoD Homolog *hlh-1*

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The myoD family of DNA binding proteins has been implicated in the control of myogenesis in a variety of organisms. Searches for homologs in the nematode *Caenorhabditis elegans* yielded only one gene, designated *hlh-1*, expressed in body-wall muscle cells and their precursors. To assess the role of *hlh-1* in *C. elegans* myogenesis, genetic deficiencies spanning the *hlh-1* locus were isolated after gamma irradiation. Embryos homozygous for these deficiencies exhibited extensive body-wall muscle differentiation, including expression of several characteristic myofilament proteins and weak contracile behavior. Thus, zygotic *hlh-1* expression was not required for body-wall muscle precursors to adopt muscle cell fates.

The myoD family of DNA binding proteins, normally expressed early in vertebrate muscle differentiation, can induce a variety of nonmuscle and premuscle cells to adopt striated muscle characteristics when ectopic expression is engineered (*1*). Whether the myoD family also initiates myogenesis during normal development can be addressed with invertebrate organisms amenable to genetic analysis: *Caenorhabditis elegans* and *Drosophila melanogaster*. To date, just one close myoD homolog has been found in each species [*hlh-1* in *C. elegans* (*2*) and *nau* in *Drosophila* (*3*)]. Similarities to vertebrate myoD family members in both sequence and muscle expression patterns suggest equivalent functions in development. Consistent with this hypothesis, the *hlh-1* product can induce myogene-

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sis when produced in mouse 10T1/2 cells (4).

The two predominant muscle types of C. *elegans* are body-wall muscles (multiple-sarcomere cells responsible for locomotion) and pharyngeal muscles (single-sarcomere cells for feeding) (5). Molecular differences between these muscle types are exemplified by expression of distinct myosin heavy chain genes [*myo-1* and *myo-2* in pharyngeal muscle; *unc-54* and *myo-3* in body-wall muscle (6)]. We have shown (2) that *hlh-1* is expressed in body-wall muscles and their precursors; no expression in pharyngeal muscle has been observed. *Caenorhabditis elegans* also has several minor muscle cell classes: intestinal, anal depressor, and sphincter muscles function in defecation and sex-specific postembryonic muscles function in egg-laying and mating (5). Although these minor muscles are single-sarcomere, they express the same myosin heavy chain isoforms as body-wall muscle (7). Detectable *hlh-1* expression was absent in minor muscles and their precursors, as assayed by both antibody staining and expression of *hlh-1::lacZ* fusions (8).

Although body-wall muscles and their precursors are the predominant site of *hlh-1* expression, two other sets of cells that are not clonal muscle precursors also contain *hlh-1* products. In early embryos at the 28-cell stage, four blastomeres [granddaughters of the multiple sclerosis (MS) founder cell] transiently show *hlh-1* expression (2, 9). Each of these blastomeres generates some body-wall muscle cells as well as other muscle and nonmuscle cells. Late in embryogenesis, six glial-like cells (GLR cells) (10) stain with antibody to *hlh-1* product and express *hlh-1::lacZ* fusion genes. GLR cells do not express muscle myosin isoforms, and no other muscle-specific product has been observed in them. GLR cells are connected by gap junctions to anterior body-wall muscle cells (10). It is thus conceivable that *hlh-1* product could enter GLR cells from the connected muscles (11). In any case, the accumulation of *hlh-1* product (at levels comparable to those in body-wall muscle precursor cells) does not appear to be sufficient to cause these cells to develop into muscles. Myogenic activation in vertebrates can be induced only within a limited range of developmental stages and conditions (12). Perhaps the MS-derived blastomeres are too early in their developmental pathways and the GLR cells too committed toward glial differentiation to respond fully to *hlh-1* expression by undergoing myogenic differentiation. A more radical interpretation of the detailed expression pattern might question accumulation of the myoD homolog as the key switch promoting myogenic commitment in C. *elegans*.

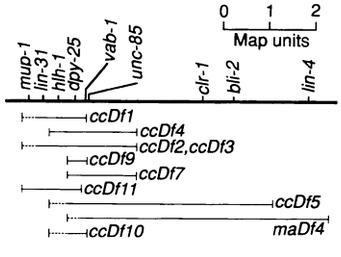

Fig. 1. Map of the left arm of chromosome II. Positions reflect genetic distances except that *hlh-1*, *mup-1* (28), and *dpy-25* are placed only as ordered relative to flanking markers. Deficiencies are aligned with genetic markers based on complementation tests. Coverage of the *hlh-1* was determined by PCR (Fig. 2). Rough approximations of molecular distance can be made from data from physical mapping (16): 400 kb from *lin-31* to *hlh-1* and 800 kb from *hlh-1* to *lin-4*.

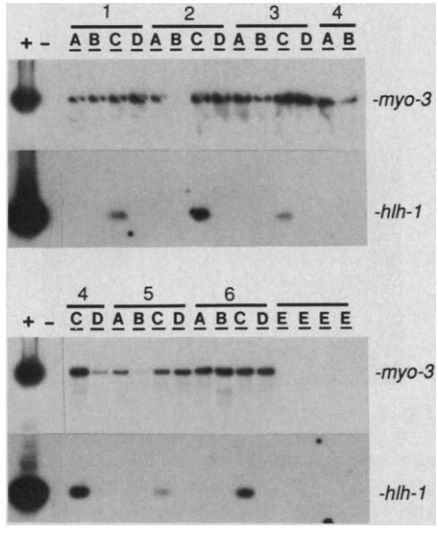

Fig. 2. PCR assays for *hlh-1* DNA sequences in arrested embryos. Single embryos (29) were frozen in 10 μl of PC2 (4 mM $MgCl_2$, 50 mM KCl, gelatin (200 μg/ml), and 10 mM tris-HCl (pH 8.4) at −70°C. After thawing, embryos were lysed and prepared for PCR as described (30). Two pairs of primers were added to each reaction: MWK93+MWK111 (specific to *hlh-1*) and MWK105+MWK106 (specific to *myo-3*). After amplification (25 cycles), samples were divided into two aliquots, resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with radioactive probes for either *hlh-1* or *myo-3*. Each lane (A through E) represents a single arrested embryo from a parent of the indicated genotype: A, *ccDf1*/+; B, *ccDf4*/+; C, *ccDf9*/+; D, *ccDf11*/+; E, bacterial strain OP50 from the same plates. Six different embryos from each strain are shown (labeled 1 through 6 on top of each gel). Positive controls (+) are 10 to 40 ng of the relevant PCR products from wild-type DNA. Negative controls (−) are lysis buffer treated identically to embryos.

Chromosomal deficiencies spanning the locus were used to examine muscle development in the absence of zygotic *hlh-1* expression. The gene *hlh-1* has been mapped by molecular techniques to chromosome II; the closest flanking markers currently assigned to both genetic and physical maps are *lin-31* and *lin-4* (2, 13). The semidominant mutation *dpy-25(e817)* provided a valuable tool in the isolation and maintenance of deficiencies in the interval; in particular, reversion of this mutation after gamma irradiation (14) yielded balanced strains heterozygous for deficiencies spanning the *dpy-25* locus (15). Nine deficiencies were aligned with the genetic map by complementation tests with recessive markers in the region (Fig. 1). Polymerase chain reaction (PCR) assays were used to test for the presence of the *hlh-1* gene in the deleted regions (Fig. 2). These data place *hlh-1* between *dpy-25* and *lin-31*.

Four representative deficiencies were selected for phenotypic analysis: *ccDf1*, *ccDf4*, and *ccDf11* include *hlh-1*, whereas *ccdf9* does not. Embryos homozygous for each of these deficiencies (deficiency homozygotes) failed to hatch but developed to a stage in which the formation of a worm with an essentially normal body plan was evident and morphological differentiation of gut, cuticle, and pharynx had occurred. Inviability of the deficiency homozygotes was expected regardless of *hlh-1* function, because gamma-ray–induced deficiencies generally span several essential genes (14). The ability of deficiency homozygotes to undergo extensive morphological differentiation was also expected, as many factors needed for embryonic development are synthesized maternally and stored in the C. *elegans* oocyte (16).

During the latter half of wild-type embryogenesis, body-wall muscle cells undergo characteristic contractions (5). Initially sporadic, these contractions become coordinated within minutes into a periodic writhing behavior (17). Null mutations in several genes encoding structural components of body-wall muscle each lead to loss of contractions (5, 18). For each of the deficiencies spanning *hlh-1*, we observed distinct body-wall muscle contractions in the arrested embryos, suggesting that some body-wall myogenesis occurred. Analyzed by time-lapse microscopy, embryos homozygous for *ccDf1* or *ccDf4* began muscle contractions at approximately the same developmental stage as wild-type embryos. Instead of progressing to coordinated writhing behavior, however, the deficiency embryos continued their relatively weak and uncoordinated contractions for several hours.

We stained homozygous deficiency embryos with antibodies to several body-wall muscle components to assess muscle structure (Fig. 3). Antibodies to the major myosin isoforms (encoded by the *unc-54* and *myo-3* genes), to paramyosin (*unc-15*), to twitchin (*unc-22*), and to vinculin (*deb-1*) all stained the deficiency embryos (*19, 20*). As in wild-type embryos, the staining pattern defined four quadrants along the anterior-posterior axis (*21*). Examined at high magnification, the *unc-54* product in the three strains with deficiencies covering *hlh-1* was poorly organized in comparison to wild-type embryos: individual sarcomeres were difficult to identify and some muscle staining extended outside the four muscle quadrants (nonquadrant staining could correspond to muscle cells with abnormal morphogenesis). We do not know if this defect in organization was due to removal of the *hlh-1* gene, deletion of flanking genes, or both (*22*). The staining was not due to cells that would become minor muscles, because only four minor muscle cells are formed during embryogenesis (*23*). Instead, the number of cells stained by each muscle antibody was close to the wild-type number of 85. Therefore, muscle development can occur in body-wall muscle precursors that lack zygotic *hlh-1* product.

Three models may address this result in the context of previous observations that suggest a central function for the myoD family in myogenic commitment. First, another myoD family member could exist in *C. elegans*. Using low stringency Southern (DNA) blots (*2, 24*), we failed to detect a close homolog of *hlh-1*. In a more general search for myoD family members, we performed PCR using three pairwise combinations of degenerate primers designed to recognize the myoD gene family (*25*). One primer pair was biased in sequence toward *myoD1* and *myf5*, a second primer pair toward *myogenin* and *myf6*, and the third pair was unbiased. Despite the biases, each primer pair amplified all four types of vertebrate myoD family members. Amplification of *C. elegans* genomic DNA with these primer pairs yielded a single major PCR product of the expected size in each case. Thirty-one clones were analyzed, 8 each from the unbiased and *myoD1/myf5* biased primers and 15 from *myogenin/myf6* primers. All 31 clones were identical in sequence to *hlh-1*. Our interpretation is that *hlh-1* is likely to be the only closely related myoD family member in *C. elegans*.

Second, the *hlh-1* product might be provided maternally. To control myogenesis, maternal myoD would need to be assymetrically partitioned or activated in only a small set of embryonic cells. Maternal myoD message has been found in *Xenopus* embryos and in the parasitic nematode *Ascaris lumbracoides* (*26*), but no functional significance has been assigned this early expression. We tested for a maternal contribution retained late in *C. elegans* embryogenesis by staining for *hlh-1* product in twofold-stage embryos homozygous for *ccDf4*. No signal was detected under conditions that would allow detection at 10% of the wild-type level. It is not possible to perform an equivalent experiment with early embryos, because deficiency homozygotes cannot at that stage be distinguished from normal siblings. To test whether the *hlh-1* product present early in embryogenesis is encoded zygotically, we introduced *hlh-1::lacZ* fusion genes from the father in crosses with wild-type hermaphrodites (*27*). The resulting embryos had β-galactosidase staining patterns identical to the initial transformed lines and similar to patterns of immunofluorescence seen with antibody to *hlh-1* product. In each myogenic lineage, staining began at the same time with the antibody as with zygotically introduced *hlh-1::lacZ*. Zygotic contributions of *hlh-1* product are thus both necessary and sufficient to explain observed expression patterns. Nonetheless, a low maternal contribution of *hlh-1* product is still possible.

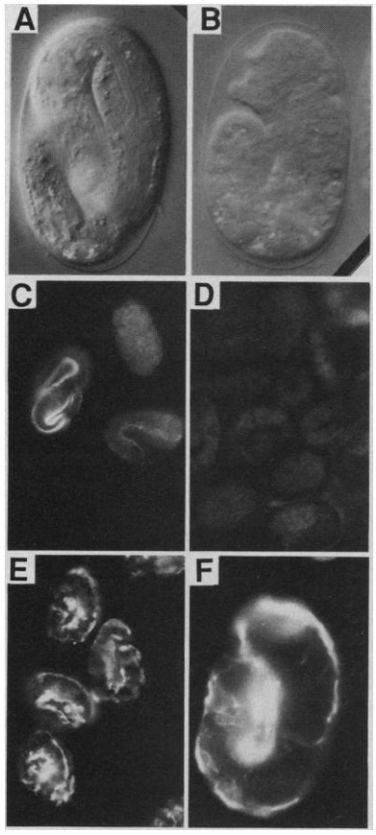

Fig. 3. Comparisons of arrested and wild-type embryos viewed by differential interference contrast microscopy and immunostaining with antibody to myosin. (**A**) Wild-type embryo viewed by differential interference contrast microscopy. (**B**) Typical *ccDf4* homozygote embryo viewed as in (A). (**C**) Wild-type: left, a late embryo at pretzel stage (*23*) with staining in well-formed muscle quadrants (one or two quadrants are in focus in any given section of the embryo); right, a comma-stage embryo with myosin synthesis evident in the one ventral muscle quadrant seen in the inner margin of the embryo; upper right, an early embryo with out-of-focus background staining. (**D**) *unc-54(e190)* embryos. This strain carries a deletion in the *unc-54* gene and fails to produce the major myosin isoform (*31*). (**E**) Staining in arrested embryos derived from *ccDf4/+* parents. (**F**) Arrested embryo from *ccDf1/+* parent. Specimens in (C) through (F) were fixed and stained with fluorescently labeled antibody to *unc-54* myosin (Ab5-8) (*19*). Each embryo is approximately 50 μm long. Staining efficiency: for a field of 21 arrested embryos derived from *ccDf9* heterozygotes, antibody to the *unc-54* product stained 20, with one embryo apparently unstained or not permeabilized. For *ccDf1*, *ccDf4*, and *ccDf11*, similar fields yielded 22/24, 23/23, and 9/9 embryos staining positively.

Third, factors other than myoD homologs might normally initiate a general program for myogenesis; the role of myoD homologs may be to enhance this program to generate diverse muscle subtypes. In *C. elegans*, body-wall and minor muscle classes could result from myogenic activation by a single myoD-independent program, which *hlh-1* modifies to produce components required for the production of multiple-sarcomere body-wall muscles.

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20. Monoclonal antibodies to *unc-54* and *myo-3* products (*19*) are specific to nonpharyngeal muscle. Monoclonal antibodies to paramyosin and vinculin were from Frances and Waterston (*7*), and polyclonal antibody to *unc-22* twitchin was from D. Moerman, G. Benian, R. Barstead, L. Schriefer, and R. Waterston [*Genes Dev.* **2**, 93 (1988)]; these antibodies stain pharyngeal as well as body-wall muscles. The minor and pharyngeal muscles have distinctive positions, not evidently displaced in deficiency homozygotes. Thus, staining in body-wall muscle quadrants by these antisera indicates muscle commitment by the cells normally destined to become body-wall muscles.
21. That the staining outlines four quadrants was confirmed by confocal fluorescence microscopy.
22. Somewhat less severe disorganization was observed in the control deficiency homozygote *ccDf9*. This difference could result either from an effect of *hlh-1* or from effects of flanking genes. Indeed, in a screen of deficiencies in different genetic regions (J. Ahnn and A. Fire, unpublished results), we have observed disorganized muscle in arrested embryos with a variety of severities ranging from complete disorganization of myofilaments to organization indistinguishable from that of wild-type embryos.
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25. Degenerate (mixed-sequence) primers used were based on highly conserved regions of the basic region and two helices: oligo MWK42 (helix 2 antisense, unbiased), cgagctcRTTNCKNARDATYTCNACYTT; oligo MWK47 (basic region, biased toward *myoD1*), cgaattcAARGCNGCNACNATGMGNGA; oligo MWK127 (basic region, biased toward *myogenin*/*myf6*), tcgaattcGTNAAYSADGCNTTYGA; and oligo MWK136 (helix 1 unbiased), tcgaattcAARGCNGCNACNYTNCGNGA. Sequences in uppercase letters were designed to recognize the myoD gene family (*1, 2*); lowercase letters represent restriction sites added for use in subsequent cloning of the PCR products. R, A or G; Y, C or T; D, A or G or T; K, G or T; S, G or C; and N, A or G or C or T.
26. Adults from a mutant strain that lacked germ cells and wild-type adults had comparable amounts of *hlh-1* mRNA. Thus, the total *hlh-1* mRNA content in the germ line (about 3000 nuclei) is at most a fraction of the total in mature body-wall muscle (95 nuclei).
27. R. Harvey, *Development* **108**, 669 (1990) (*Xenopus*); M. Krause, unpublished results (*Ascaris*).
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29. To obtain populations of embryos homozygous for each deficiency, we crossed the original strains [*Df*/*dpy-25*(*e817*)] with wild-type males and selected non-dumpy cross progeny. These *Df*/+ animals were then selfed, and the arrested embryos stained and prepared for PCR. If all dead embryos were indeed deficiency homozygotes, then just 25% would be expected to arrest. This was observed for *ccDf1* and *ccDf4*. For *ccDf11*/+, approximately half of the embryos arrested; hence, only about 50% of these arrested embryos were *ccDf11* homozygotes.
30. R Barstead, L. Kleiman, R. Waterston, *Cell Motil. Cytoskeleton* **20**, 69 (1991).
31. H. Epstein, R. Waterston, S. Brenner, *J. Mol. Biol.* **90**, 291 (1974). Note that *unc-54* expression is exclusively zygotic: homozygous *unc-54* null embryos failed to express any *unc-54* myosin even if derived from an *unc-54*(o)/+ mother (*24*).
32. We thank J. Priess for guidance, J. Thomas for help identifiying GLR cells, and C. Mello, A. Shearn, C. Vinson, T. Schedl, S. Tapscott, and S. White-Harrison. Some nematode strains were from the *Caenorhabditis* genetics center, funded by the NIH Center for Research Resources. Supported by NIH grants to A.F. and H.W. M.K. is a W. C. Gibson Fellow of the Muscular Dystrophy Association. A.F. is a Rita Allen Foundation Scholar.

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Lithium-Sensitive Production of Inositol Phosphates During Amphibian Embryonic Mesoderm Induction

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Mesoderm induction and body axis determination in frog (*Xenopus*) embryos are thought to involve growth factor–mediated cell-cell signaling, but the signal transduction pathways are unknown. Li^+, which inhibits the polyphosphoinositide (PI) cycle signal transduction pathway in many cells, also disrupts axis determination and mesoderm induction. Amounts of the PI cycle–derived second messenger, inositol 1,4,5-trisphosphate, increased during mesoderm induction in normal embryos; addition of Li^+ inhibited the embryonic inositol monophosphatase and reversed this increase. Embryonic PI cycle activity thus shows characteristics that indicate it may function in mesoderm induction and axis determination.

Among the earliest developmental decisions made by vertebrate embryos are those that determine the prospective germ layers (endoderm, mesoderm, and ectoderm) and body axes (dorsoventral and anteroposterior). For embryos of the frog, *Xenopus laevis*, experiments involving either cell transplantation or imposition of cell-impermeable filters between blastomeres indicate that mesoderm induction and its corollary, axis determination, begin at about the 32- to 64-cell stage (*1*) and involve secreted diffusible factors that are released from prospective endodermal ("vegetal") cells and act on their overlying equatorial and "animal hemisphere" neighbors (*2, 3*). Three such signals have been proposed to exist (*4*), differing across the prospective dorsoventral axis.

Dorsal and ventral mesoderm-inducing signals may include homologs of the growth factors activin and basic fibroblast growth factor (*3, 5*), but whatever the inducing factors may be, the intracellular signal transduction pathways used in mesoderm induction remain unknown. Involvement of the PI cycle has been suggested on the basis of the teratogenic effect on mesoderm induction of the PI cycle inhibitor Li^+ (*6, 7*). Activation of the PI cycle by calcium-mobilizing hormones or growth factors leads to hydrolysis of the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, generating inositol 1,4,5-trisphosphate (IP_3), which triggers release of Ca^{2+} from the endoplasmic reticulum into the cytosol, and diacylglycerol, the endogenous activator of protein kinase C (*8*). Lithium inhibits the inositol mono- and bisphosphatases that recycle IP_3 to *myo*-inositol, the precursor of the inositol phospholipids (*9*). Injection of Li^+ into a ventral vegetal cell of the 32-cell *Xenopus* embryo redirects the developmental fates of that cell's progeny toward dorsal mesodermal derivatives and dorsal organizer tissue (*6, 10*). This effect is prevented by co-injection of *myo*-inositol, whereas *epi*-inositol (an isomer not used in the PI cycle) has no effect (*6*). Such isomer-specific rescue implicates the PI cycle as the target of Li^+ and suggests PI cycle involvement in normal mesoderm induction and axis determination. We investigat-

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